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## Contribution of *Enterococcus faecalis* to urinary tract infection

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Graduate Program in Microbiology and Immunology  
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of  
Philosophy  
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## Abstract

The purpose of this thesis was to increase understanding of enterococcal urinary tract infection (UTI), in particular, the response of *Enterococcus* to antibiotic prophylaxis *in vitro* and *in vivo* and enterococcal communication with the bladder.

We studied the *in vitro* effects of trimethoprim-sulfamethoxazole (TMP/SMX) and nitrofurantoin, two of the most commonly used antibiotic treatments for the management of both UTI and recurrent UTI (RUTI), on *Enterococcus faecalis* attachment to urothelial cells. In doing so, we documented increases in bacterial attachment at growth inhibitory concentrations of nitrofurantoin, but not TMP/SMX. This increased virulence did not correlate with increased expression of virulence factors but was correlated with increased expression of three putative genes.

We then explored whether this corresponded to alterations in bacterial communities throughout antibiotic prophylaxis for paediatric patients with RUTI. Bacterial culture results indicated uropathogens were present in the urine of children with and without a history of RUTI and that antibiotic prophylaxis induced a transient decrease in uropathogen load. Interestingly, none of our patients were experiencing symptomatic UTI at the time of urine sample collection, yet a significant proportion of midstream urine samples met the clinical threshold for UTI, indicating these patients had asymptomatic bacteriuria (ABU). Further, *E. faecalis* bacterial load was positively correlated with non-*E. coli* uropathogens, suggesting some patients may be pre-disposed to polymicrobial UTI.

To elucidate mechanisms by which enterococci can mask the host's perception of UTI, which may also contribute to the polymicrobial nature of *E. faecalis* UTI, we completed targeted metabolomics of neuroactive molecules *in vitro* under conditions mimicking the bladder environment. Our results suggest *Enterococcus* may produce tyramine in the bladder at concentrations that are likely to have a physiological effect on both urothelial cells and cohabiting bacteria.

Our data raises questions about the application of nitrofurantoin to enterococcal UTI and the efficacy of antibiotic prophylaxis for RUTI. Further, our clinical and *in vitro* data suggest *E. faecalis* may contribute more to polymicrobial UTI than previously thought. Indeed,

enterococcal production of tyramine may explain the high incidence of enterococci in not only polymicrobial UTI but also other infections.

## Keywords

*Enterococcus faecalis*, urinary tract infection, recurrent urinary tract infection, antibiotic prophylaxis, paediatric urology.

## Co-Authorship Statement

Samantha Whiteside predominantly carried out the experiments and data analysis within this thesis with the supervision of Jeremy Burton and Gregor Reid. Samantha Whiteside primarily wrote the chapters presented herein; exceptions are listed below:

### **Chapter 1: General introduction**

The section on the microbiome of the urinary tract (Section 1.2) was conceived by Samantha Whiteside, Jeremy Burton, and Gregor Reid. Hassan Razvi and Sumit Dave provided clinical input.

### **Chapter 2: Nitrofurantoin increases bacterial attachment to urothelial cells**

Samantha Whiteside, Jeremy Burton, and Gregor Reid conceived the experiments. Aidan Shair completed the PCR screen and ibuprofen growth curves; Samantha Whiteside carried out the remainder of the assays and performed data analysis.

### **Chapter 3: Longitudinal study of the bacterial communities in urine from children with RUTI**

Samantha Whiteside, Jeremy Burton, Gregor Reid, and Sumit Dave conceived and designed the study. Sample collection, urinalysis, and patient follow-up was completed by the paediatric urology clinic staff. Patient clinical files were reviewed by both Samantha Whiteside and Peter Wang. Urine sample processing, including bacteriological culture and DNA extraction were performed by Samantha Whiteside. Kait Al completed the DNA amplification and submitted samples for sequencing. Microbiota analysis was performed by Samantha Whiteside, using the pipeline designed by Gregory Gloor and Jean Macklaim, whom also provided input. Shannon Seney performed cytokine multiplex assays, which were subsequently analysed by Samantha Whiteside.

### **Chapter 4: Enterococcal production of neuroactive substances**

Samantha Whiteside, Jeremy Burton, and Gregor Reid conceived the experiments. Fatemah Mousavi and Janusz Pawliszyn optimised the SPME-LC-MS/MS methodology for microbial



metabolomics. Samantha Whiteside performed the GABase experiments, all sample preparation, and metabolite extraction for exploratory metabolomics. Fatemeh Mousavi completed the exploratory LC-MS/MS and Samantha Whiteside analysed the data. Extraction and identification of metabolites for quantitative metabolomics was performed by Sofia Lendor and Janusz Pawliszyn, data analysis was completed by Samantha Whiteside.

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To my supervisors, Jeremy and Gregor, thank you for your guidance and support over the years. Together you foster an environment of scientific creativity, friendship, and instill within each of us the desire to explore the unknown. You gave even my most outrageous theories consideration and I cannot thank you enough for the opportunities I have had within your research group.

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To my family, you don't always understand what I do, or why I do it, yet seem to appreciate telling people that over my research career I have developed a fascination for faeces in undergrad and urine in grad school. I am incredibly grateful for the love within our family and the times we've shared together. There is no one else I would want to argue with about "North African Trade Oranges," torpedo trajectories through islands, or whose turn it is to host the next Family Scotch Club. You keep me honest and grounded.

Brandon; I'm not sure how I tricked you into it, but you've committed to spending the rest of your life with me and I cannot wait to see where we go. You remind me to breathe, appreciate the moment, and push me to be a better person. I would probably be living in a gutter, if it were not for your, love, patience, support, compassion, sense of adventure, and ability to laugh both at me and with me.

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## Abbreviations

2-AG – 2-Arachidonylglycerol

3-MT – 3-Methoxytyramine

5-HT – Serotonin

ABU – Asymptomatic bacteriuria

Ace – Adhesion to collagen of *Enterococcus faecalis*

ACh – Acetylcholine

AChE – Acetylcholinesterase

ACP – Acyl carrier protein

ACTB –  $\beta$ -actin

AhrC – ArgR family transcriptional regulator

AJ – Adherens junction

Ang-II – Angiotensin II

ANOVA – Analysis of variance

AS – Aggregation substance

Asa1 – Aggregation substance protein 1

ASV – Amplicon sequencing variant

ATCC – American Type Culture Collection

ATP – Adenosine triphosphate

AU – Artificial urine

B2M –  $\beta$ 2-microglobulin

BBD – Bladder and bowel dysfunction

BDNF – Brain-derived neurotrophic factor

BH – Brain-heart infusion

BHYE – Brain heart infusion supplemented with 0.25% yeast extract

BOO – Bladder outlet obstruction

CAMHB – Cation-adjusted Mueller Hinton broth

cAMP – Cyclic adenosine monophosphate

CAUTI – Catheter-associated urinary tract infection

CBA – Columbia blood agar with 5% defibrinated sheep's blood

CDC – Centre for Disease Control

CDH1 – E-cadherin

cDNA – Complementary DNA

CFU – Colony forming units

CGRP – Calcitonin gene-related peptide

CIBU – 2-Carboxyibuprofen

COX-1 – Cyclooxygenase-1, constitutive

COX-2 – Cyclooxygenase-2, inducible

CPPS – Chronic pelvic pain syndrome

CPS – Canadian Paediatric Society

CTNNA1 –  $\alpha$ -catenin

CTNNB1 –  $\beta$ -catenin

CylL – Cytolysin

CylL<sub>L</sub> – Cytolysin structural subunit, large

CylL<sub>S</sub> – Cytolysin structural subunit, small

DA – Dopamine

DMF – N,N-dimethylformamide

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DTT – Dithiothreitol

DVSS – Dysfunctional voiding scoring system

Ebp – Endocarditis-and-biofilm-associated pili

EbpA – Endocarditis-and-biofilm-associated pilus, subunit A

EbpB – Endocarditis-and-biofilm-associated pilus, subunit B

EbpC – Endocarditis-and-biofilm-associated pilus, subunit C

ECM – Extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

EF0404 – Gene locus for a putative nitroreductase

EF0420 – Gene locus for a putative drug resistance transporter of the MFS

EF0648 – Gene locus for a putative nitroreductase

EF0655 – Gene locus for a putative nitroreductase

EF0972 – Gene locus for a putative DNA repair exonuclease

EF1181 – Gene locus for a putative nitroreductases

EF1370 – Gene locus for a putative drug resistance transporter of the MFS

EF1413 – Gene locus for a MsrC-like protein

EF1732 – Gene locus for putative ABC transporter ATP-binding peptidase of the MDR family

EF1733 – Gene locus for putative ABC transporter ATP-binding peptidase of the MDR family

EfaA – *Enterococcus faecalis* endocarditis antigen

EfbA – PavA-like fibronectin-binding protein of *E. faecalis*

EmeA – Enterococcal MDR efflux

ENS – Enteric nervous system

EOS – Ethanolamine-*O*-sulphate

EPI – Epinephrine

EQUC – Expanded quantitative urine culture

ESBL – Extended-spectrum beta-lactamase

Esp – Extracellular surface protein

FabF-1 – 3-oxoacyl-acyl carrier protein synthase

FabI – Enoyl-acyl carrier protein reductase

FabZ-1 – (3R)-hydroxymyristoyl-acyl carrier protein dehydratase

FsrA – *E. faecalis* regulator, response regulator

FsrABC – Fsr-quorum sensing system

FsrB – *E. faecalis* regulator

FsrC – *E. faecalis* regulator, histidine kinase

FV – First-void urine

GABA -  $\gamma$ -aminobutyric acid

GABA-T – GABA transaminase

GABase – Commercial preparation of GABA-T and SSDH

GAG – Glycosaminoglycan

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GelE – Gelatinase

GHB -  $\gamma$ -hydroxybutyric acid

Gls24 – General stress protein 24

GlsB – General stress protein B

GrvR – Global regulator of virulence, DNA-binding response regulator

GrvRS – Global regulator of virulence system

GrvS – Global regulator of virulence, sensor histidine kinase

HIS – Histamine

HLB – Hydrophilic-lipophilic balanced

IBC – Intracellular bacterial communities

IBU - Ibuprofen

IC – Interstitial cell

IC/BPS – Interstitial cystitis/bladder pain syndrome

ICC – Interstitial cells of Cajal

IDO – Idiopathic detrusor overactivity

IFN $\gamma$  – Interferon- $\gamma$

IFTA – Interstitial fibrosis and tubular atrophy

IL-10 – Interleukin-10

IL-1 $\beta$  – Interleukin-1 $\beta$

IL-2 – Interleukin-2

IL-6 – Interleukin-6

IL-8 – Interleukin-8

iNOS – Inducible nitric oxide synthase

IRIS – Italian renal infection study group in children

JUP -  $\gamma$ -catenin (junction plakoglobin)

KO – Knockout (genetic)

L-type Ca<sup>2+</sup> - Long-lasting, voltage dependent calcium channel

LB – Luria-Bertani

LOD – Limit of detection



LOQ – Limit of quantitation

LP – Lamina propria

LPS – Lipopolysaccharide

LUT – Lower urinary tract

LUTS – Lower urinary tract symptoms

mAChR – Muscarinic acetylcholine receptor

MALDI-TOF MS – Matrix-assisted laser desorption/ionization-time of flight mass spectrometry

MAO – Monoamine oxidase

MarR – Multiple antibiotic resistance

MDR – Multi-drug resistance

MFS – Major facilitator superfamily

MIC – Minimal inhibitory concentration

mL – milliliter ( $10^{-3}$  litre)

MLCK – Myosin light chain kinase

MLCP – Myosin light chain phosphatase

MOI – Multiplicity of infection

MS – Midstream urine

MsrC – Macrolide and streptogramin B resistance, protein C

nAChR – Nicotinic acetylcholine receptor

NADA – N-arachidonoyl dopamine

NADP<sup>+</sup> – Nicotinamide adenine dinucleotide phosphate

NBD – Neurogenic bladder dysfunction

NE – Norepinephrine

NGF – Nerve growth factor

Nitro – Nitrofurantoin

NKA – Neurokinin A

NMR – Nuclear magnetic resonance

nNOS – Neuronal nitric oxide synthase

NO – Nitric oxide

NOS – Nitric oxide synthase

NPY – Neuropeptide Y

NSAID – Non-steroidal anti-inflammatory drug

OAB – Overactive bladder

Obs – Clinical observation

OD – Optical density

OHIP – Ontario health insurance plan

OhR – Organic hydroperoxide resistance protein

P2X – Purinergic ATP-gated P2X receptor cation channel

P2Y – Purinergic G protein-coupled P2Y receptor

PAN – Polyacetonitrile

PavA – Adherence and virulence protein A

PBS – Phosphate buffered saline

PC – Principle component

PCA – Principle component analysis

PCR – Polymerase chain reaction

PDGFR $\alpha$  – Platelet derived growth factor receptor- $\alpha$

PEG3350 – Polyethylene glycol 3350

PG – Prostaglandin

PGP9.5 – Protein gene product 9.5

PKA – Protein kinase A

POP – Pelvic organ prolapse

PRIVENT – Prevention of recurrent urinary tract infection in children with vesicoureteral reflux and normal renal tracts

PS-DVB-WAX – Polystyrene-divinylbenzene weak anion exchange

qPCR – Quantitative PCR

RecA – Recombinase A

RIVUR – Randomized intervention for children with vesicoureteral reflux

RnjB – Rnase J2

RPMI – Roswell Park Memorial Institute medium 1640

rRNA – Ribosomal ribonucleic acid

RUTI – Recurrent urinary tract infection

s/n – Signal-to-noise

SalB – Secreted lipase

SCI – Spinal cord injury

SlyA – MarR family transcriptional regulator

SMC – Smooth muscle cell

SP – Substance P

SPME-LC-MS/MS – Solid phase microextraction coupled with liquid chromatography-tandem mass spectrometry

SprE – Extracellular serine protease

SSDH – Succinate semialdehyde dehydrogenase

STI – Sexually transmitted infection

SUI – Stress urinary incontinence

T2DM – Type 2 diabetes mellitus

TAAR1 – Trace amine-associated receptor 1

TAAR2 – Trace amine-associated receptor 2

TER – Transepithelial electrical resistance

TMP/SMX – Trimethoprim-sulfamethoxazole (Septra)

TNF $\alpha$  – Tumor necrosis factor alpha

TRP – Transient receptor potential

TRPV1 – Transient receptor potential cation channel, subfamily V, member 1

TRPV4 – Transient receptor potential cation channel subfamily V member 4

UC – Umbrella cell

UI – Urinary incontinence

UP – Uroplakin

UPEC – Uropathogenic *Escherichia coli*

UTI – Urinary tract infection

UUI – Urgency urinary incontinence

VACHT – Vesicular acetylcholine transporter

VanSR – Vancomycin resistance system

VCUG – Voiding cystourethrogram

VIP – Vasoactive intestinal peptide

VRE – Vancomycin resistant *Enterococcus*

VUR – Vesicoureteral reflux

WHO – World Health Organization

$\alpha$ -AR –  $\alpha$  adrenergic receptor

$\beta$ -PEA -  $\beta$ -Phenylethylamine

$\beta$ 3-AR –  $\beta$ 3 adrenergic receptor

$\mu$ L – microliter ( $10^{-6}$  litre)

## Chapter 1 \*

### 1 General Introduction

The urinary tract encompasses the kidneys, ureters, bladder and urethra. Functionally, the kidney plays a role in blood pressure homoeostasis, secretion and absorption of molecules and ions, and blood filtration, while the bladder is often simplified to the storage and voiding of urine. However, signalling to control and transition between these states is much more complex than traditionally acknowledged and incompletely understood. Indeed, there appears to be multiple levels of sensation and action within the urinary tract, and this has become a broadly studied area in recent decades.

While bacterial interactions with these systems are better studied in the context of the enteric nervous system and brain, sparse research has gone into understanding the bacterial interface with the sensory and motor systems of the urinary tract. The recent discovery of bacterial communities native to urine, however, has opened such avenues of research.

Herein we explore the role of the urinary microbiota in paediatric disease, the role of antibiotics in the progression of urinary tract infections (UTIs), and the potential for bacteria to interact with the signalling systems of the urinary bladder.

#### 1.1 Functional anatomy of the urinary tract

Bacteria are suspected of modulating bladder and host behaviour through multiple modalities; comprehension of the extent and downstream effects of these interactions during states of health and disease requires a basic understanding of functional anatomy. The urinary tract is divided into the upper urinary tract, consisting of the kidneys and

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ureters, and lower urinary tract (LUT), comprising the urinary bladder and urethra. The urinary bladder is a hollow, smooth muscle organ that functions to store and release soluble waste from the kidney. Anatomically, the bladder is divided into the bladder body, the region above the ureteral orifices and the bladder base, which is inclusive of the trigone and bladder neck (Figure 1-1)<sup>1</sup>. This is important to note, as the tissue structure and innervation of the bladder varies between these two regions.

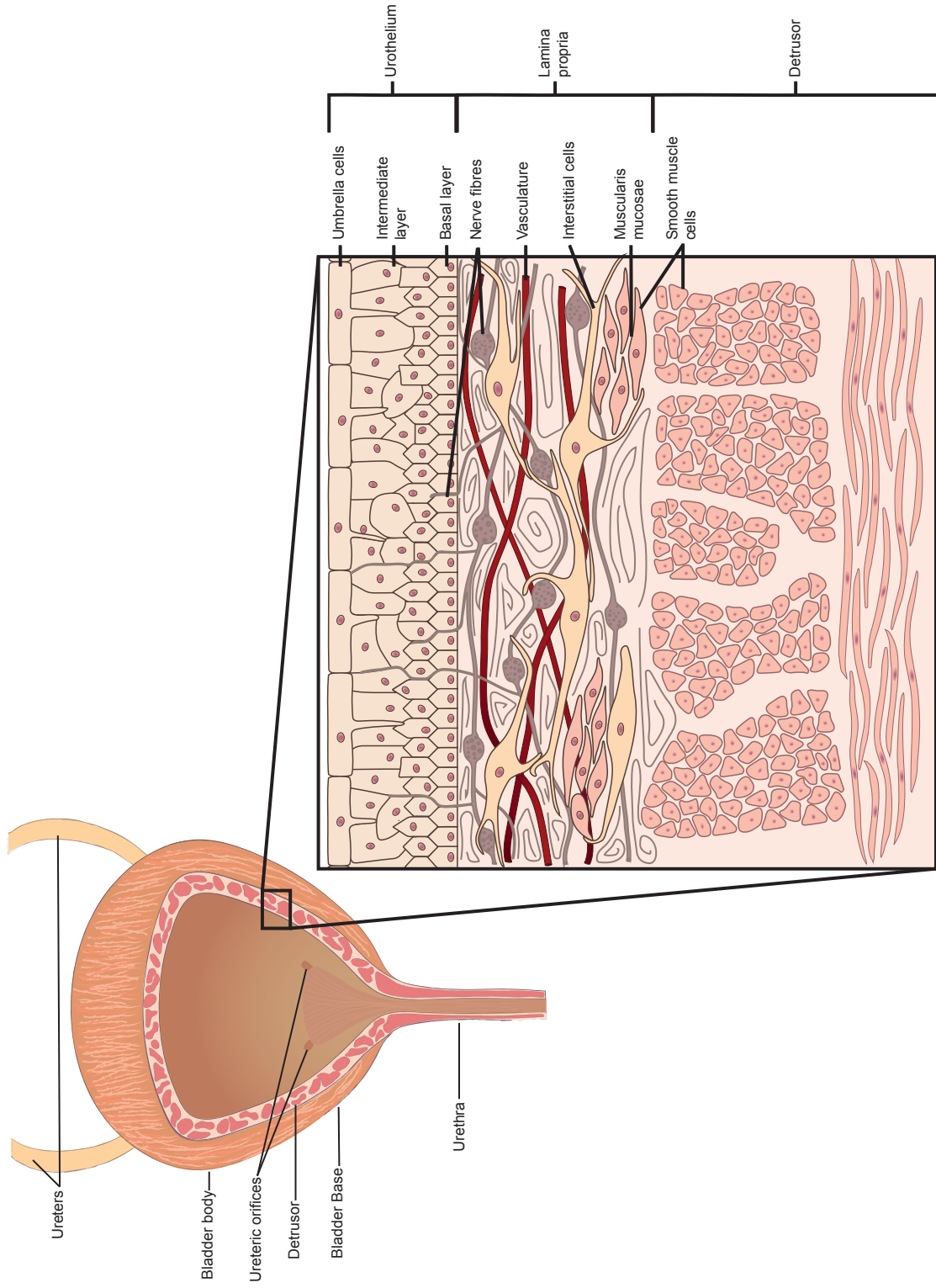
### 1.1.1 The bladder wall

The bladder wall is composed of three layers; the mucosa, detrusor, and the adventitia/serosa (Figure 1-1). The mucosal layer consists of the urothelium, which lines the bladder lumen, and the lamina propria. The detrusor muscle, incorporates three smooth muscle compartments, of which the smooth muscle cells of the inner and outer layer are oriented longitudinally and the middle circularly; the layers run longitudinally, transversely and obliquely<sup>1,2</sup>. The muscle layer is covered by the serosa on the superior and lateral surfaces, and the retroperitoneal surface is surrounded by adventitia.

#### 1.1.1.1 Mucosa

##### 1.1.1.1.1 Urothelium

The luminal lining of the bladder, the urothelium, creates an impermeable, non-adherent barrier that forms an interface between the urinary space and underlying connective, nervous, vasculature, and muscular tissues. Given the presence of bacteria in urine from functionally healthy and diseased bladders, this tissue is the site of direct bacterial influence, mediating interactions with the sensory, motor, and immune systems. The urothelium also lines the renal pelvis, ureter, upper urethra, and glandular ducts of the prostate in males; these different anatomical locations represent different cell lineages and therefore differ in functional and biochemical properties<sup>3</sup>. Notably, the renal pelvis and ureter are mesoderm-derived, while the bladder, bladder neck and urethra are endoderm-derived<sup>4,5</sup>.





**Figure 1-1. Bladder anatomy and mucosal structures.**

A smooth muscle organ, the bladder has a hollow sack-like structure that is anatomically divided into the bladder body and base. Urine, which is produced by the kidneys (not shown), passes through the ureters to the bladder for storage until micturition, at which point it is excreted from the body through the urethra. Tissue layers of the bladder include the mucosa, which consists of the urothelium and lamina propria (inset), detrusor, and adventitia/serosa (not shown). As a transitional epithelium, the urothelium consists of three general layers; the basal and intermediate layers, as well as a terminally differentiated layer of umbrella cells. The lamina propria (LP) lies beneath the urothelium; it is both highly innervated and vascularized. Interstitial cells and muscularis mucosae are also found within the LP, and these have been suggested to aid in communication between the urothelium, detrusor, and nervous system.

A transitional epithelium, the urothelium is subdivided into three general layers, the basal layer, which is attached to the basement membrane, an intermediate layer, and a superficial apical layer of umbrella cells (UC). Humans are thought to have multiple intermediate layers, while rodents have a minimum of three, resulting in a tissue thickness between three and seven cell layers<sup>6,7</sup>.

Umbrella cells are large hexagonal cells that create the bladder's physical barrier against infection and toxic substances found in urine<sup>3,6,8</sup>. Maintenance of this watertight barrier function is multifactorial, involving tight junctions between cells, and uroplakin (UP)-plaques as well as a unique lipid layer on the apical surface<sup>8</sup>. Indeed, the apical surface of these cells is unusually rich in cholesterol, phosphatidylcholine, phosphatidylethanolamine, and cerebroside<sup>9</sup>. The cell surface is further covered by a glycosaminoglycan (GAG) layer, which has also been suggested to play a role in the bladder's barrier function<sup>10,11</sup>; however, this is controversial<sup>1,12</sup>. Collectively, these properties create one of the most effective barriers of any biomembrane<sup>3,13</sup>.

Umbrella cells are truly exceptional cells in that they maintain their barrier properties while undergoing dramatic changes in shape. For instance, in the empty bladder UCs form globular rectangles, yet when the bladder is full, they take on a flattened, flagstone-like shape. Such hypercompliance is conferred in part by UP-plaques and increased vesicular traffic during bladder filling, which physically adds membrane to the superficial surface<sup>8,13-15</sup>. This increased membrane traffic has also been linked to regulation of receptor and channel expression profiles of urothelial cells, including those of the apical and intermediate layers<sup>16</sup>.

The formation of UP-plaques, which cover about 90% of the UC apical cell surface and form the asymmetric unit membrane, serves as a differentiation marker for UCs<sup>3</sup>.

Indeed, UP proteins are highly expressed in the urothelium, yet expression in normal non-urothelial cells is uncommon<sup>17</sup>. Four UP proteins have been described, UPIa, UPIb, UPII, and UPIIIa<sup>3</sup>, each demonstrating a high degree of evolutionary conservation<sup>18,19</sup>. Differential expression is also noted within human urothelial layers, with the expression of UPIIIa, UPIa, and UPII occurring only in umbrella cells<sup>17,20</sup>. Expression of these

proteins is taken advantage of by uropathogens, such as uropathogenic *Escherichia coli* (UPEC) and *Klebsiella pneumoniae*, which bind UPs to initiate adhesion<sup>21</sup>.

Concurrently, the interaction of UPEC FimH with UPIa induces UPIIIa phosphorylation followed by the elevation of intracellular calcium<sup>22,23</sup>, suggesting UPs modulate the early host response to infection. Unsurprisingly, UP deficiency contributes to a compromised urothelial permeability barrier and global urinary tract anomalies<sup>13,24-27</sup>.

A number of factors modulate the urothelium's barrier function, including tissue pH, mechanical or chemical trauma, hormonal changes and of relevance here, bacterial infection<sup>6,16</sup>. For example, bacterial contact with the UC apical surface induces exfoliation of UCs and loss of defined intercellular junctions at the apical surface, corresponding with a decrease in transepithelial electrical resistance (TER) relative to controls<sup>28,29</sup>. Of note, UTI, interstitial cystitis/bladder pain syndrome (IC/BPS), spinal cord injury (SCI), and senescence are associated with changes in the urothelium, which may explain some the associated bladder dysfunction<sup>28,30-34</sup>.

In the healthy bladder, urothelial integrity is maintained through a complex process of migration, proliferation, and differentiation, yet some studies propose proliferation takes precedence over differentiation<sup>7,31</sup>. This was recently highlighted by a SCI model, wherein researchers noted signs of incomplete differentiation persisting at 28 days post-injury<sup>7</sup>. Similar apical cell layer characteristics including; small cell size, lack of UC differentiation markers, and altered tight junction integrity, have been linked to increased apical surface stiffness, decreased TER, and increased permeability to both water and urea<sup>13,29,31,33</sup>. Interestingly, Scott Hultgren's group has documented dysfunctional remodelling of the bladder mucosa in mice following experimentally induced chronic UTI<sup>28</sup>. The Hultgren model relies on two subsets of mice, those that spontaneously resolve chronic infection (resolved) and those that are unable to clear the infection and are subsequently treated with antibiotics (sensitised). At 8-weeks post-infection, both resolved and sensitised mice display mucosal remodelling. However, when challenged with bacteria, resolved mice generally develop a mild infection that is rapidly cleared, while sensitised mice develop severe acute and chronic UTI. Furthermore, in sensitised mice the superficial cells remain up to 20-times smaller than

naïve UCs and express UPIIIa, but not Krt20, another marker of terminal UC differentiation. Acute infection studies suggest mucosal remodelling begins within 5 hours of UPEC infection<sup>29</sup>. Notably, small superficial cells are also found in UPII and UPIIIa knockout (KO) mice, concomitantly with increased cell stiffness and permeability to both water and urea<sup>13,24-26</sup>.

In humans, staining of UPIIIa from the urothelium of healthy individuals is uniform across the apical surface; however, in patients with recurrent UTI (RUTI) and vesicoureteral reflux (VUR) staining for UPIIIa is either inconsistent or absent<sup>34</sup>. Interestingly, UPIIIa KO mice are considered an appropriate model for human VUR, yet clinical studies have failed to link mutations in UPIIIa to primary VUR<sup>35,36</sup>. This suggests that the loss of UPIIIa expression along the apical surface in VUR patients may be secondary to infection-induced remodelling; particularly given the high incidence of UTI in this population. In general, study of the UP proteins in human disease is limited, the majority of research identifies UP proteins as effective markers of bladder cancer metastasis<sup>3</sup>.

Thus, following urothelial damage, the barrier does not function optimally, even though the damage has been physically repaired. As one of the slowest cycling epithelia<sup>3,37</sup>, such damage may have long-lasting effects; predisposition to infection, as seen in the aforementioned mouse model, or interference with bladder sensation and detrusor contraction. Further, the similarities in mucosal remodelling associated with SCI and previous UTI may provide an explanation for the high rate of UTI in these populations.

In addition to its function as a physical barrier, the urothelium is recognised as metabolically active and an active contributor to the sensory functions of the bladder. Notably, the urothelium responds to both chemical and mechanical stimuli and communicates with nerves, as well as smooth muscle, interstitial, and inflammatory cells<sup>6,38</sup>. Alterations in uroepithelial receptor expression and release of neurotransmitters, such as adenosine triphosphate (ATP) and acetylcholine (ACh), has been linked to bladder pathology<sup>16</sup>.

### 1.1.1.1.2 Lamina Propria

Sometimes referred to as the suburothelium, the lamina propria (LP) lies between the basement membrane of the urothelium and the detrusor muscle (Figure 1-1). Within the LP, extracellular matrix (ECM) surrounds a variety of cell types including fibroblasts, adipocytes, interstitial cells (IC), afferent and efferent nerve fibres, as well as a rich vascular network, lymphatic channels, and smooth muscle fascicles (muscularis mucosae)<sup>6,39-42</sup>. The ECM acts a structural scaffold of proteins, proteoglycans, and glycosaminoglycans that provides support and signalling to bladder cells. Collagen I and III make up most of the protein mass within the ECM at a ratio of 3:1<sup>40</sup>. When the bladder is empty, collagen III is distributed in a loose network of fibres without uniform orientation; however, during bladder filling these fibres rearrange to collectively reorient parallel to the urothelium<sup>40</sup>.

While the functional role of the LP is undefined, researchers have suggested it may play a capacitance role, such that it determines bladder compliance and enables adaptive change to accommodate increasing bladder volume<sup>40</sup>. Cells of the LP have also been shown to accelerate the growth of urothelial and smooth muscle cells *ex vivo* in both a juxtacrine and paracrine manner<sup>43</sup>. The function and characterisation of many cells within the LP remains speculative, and in some cases controversial.

The presence of muscularis mucosae was first described in the human bladder and confirmed in the guinea pig<sup>42,44,45</sup>; these muscle bundles, however, have not been found in the rat, rabbit, cat, or dog<sup>42</sup>. While not well defined, they are described as irregularly arranged muscle bundles containing small-diameter smooth muscle cells rich in nonspecific cholinesterase and glycogen. The smooth muscle cells are morphologically and histochemically distinct from detrusor muscle and innervated by acetylcholinesterase (AChE) positive nerve fibres<sup>42,46</sup>.

Alternatively, bladder ICs are fairly well studied, although a lack of consensus on nomenclature hinders functional study comparison and contributes to the controversy surrounding these cells. In general, spindle-shaped bladder cells are categorised as ICs,

interstitial cells of Cajal (ICC), interstitial Cajal-like cells, myofibroblasts, and in some cases telocytes. Differences in IC response and immunoreactivity between species aggravate the controversy<sup>38</sup>. Throughout the literature, however, one facet has remained constant, the use of the gut as a model to study bladder ICs.

In the gut, ICCs perpetuate signals between enteric nerves and smooth muscle cells (SMCs), serving as a pacemaker and mediator of neurotransmission<sup>47</sup>. Interstitial cells of Cajal are found in close proximity to enteric nerve fibres and are capable of generating and conducting electrical signals with one another and SMCs via gap junctions<sup>47</sup>. A similar function has been proposed for bladder ICs, yet this is a source of contention<sup>45,47,48</sup>. In the human bladder, ICs are identified in the lamina propria and detrusor based on immunoreactivity to vimentin, proto-oncogene c-Kit, and/or platelet-derived growth factor receptor- $\alpha$  (PDGFR $\alpha$ ), in addition to morphological properties, such as spindle or stellate morphology<sup>46,49-54</sup>. Notably, mast cells are also c-Kit<sup>+</sup> and have been described in the human bladder, yet these cells exhibit a rounded shape<sup>46,55</sup>. Throughout the LP, ICs create a mesh-like network and are also found as elongated cells along the blood vessels and muscularis mucosae<sup>49</sup>. In addition to the LP, ICs are found within the detrusor, creating two populations of bladder IC that are morphologically and histochemically distinct<sup>46,54,56</sup>. Interstitial cells in the bladder demonstrate a number of electrical and structural characteristics that may allow them to act as an electrical network, including a resting membrane potential of -60mV, the presence of gap junctions, and co-localization with nerve fibres<sup>46,49,51,52,57</sup>.

Similar to the gut, human bladder IC are in contact with nerve fibres, as characterised by antibody labelling for protein gene product 9.5 (PGP9.5)<sup>49</sup>, neuronal ubiquitin hydrolase that is often used as a general neuronal marker, and vesicular acetylcholine transporter (vAChT)<sup>46</sup>, which is cholinergic nerve specific. This property was also demonstrated in the detrusor, where ICs associate with vAChT-labelled nerve fibres<sup>46</sup>. Interestingly, other groups have documented expression of the muscarinic acetylcholine receptor (mAChR) subtypes M<sub>2</sub> and M<sub>3</sub> by ICs in the LP of human, rat, and guinea pig bladder<sup>50,58,59</sup>. Further, experiments in guinea pig ICs demonstrated the functional activity of these receptors and the ability of ICs to transfer the signal between adjacent

cells<sup>60</sup>. Thus, ICs are found in close contact with cholinergic nerve fibres and express functional receptors for the cholinergic effector, acetylcholine, which suggests ICs are capable of receiving and modulating cholinergic nerve input.

Interstitial cells may also function as a regulator of detrusor excitability during bladder filling, as has been proposed in the mouse. Platelet-derived growth factor receptor- $\alpha$  immunoreactive cells are located throughout the LP and detrusor, creating a loose network where some, but not all cells, are in contact with PGP9.5<sup>+</sup> nerve fibres<sup>57</sup>. These cells express small conductance calcium-activated potassium channels, which, when exposed to agonists, induced significant hyperpolarisation in isolated cells<sup>61</sup>.

Administration of ATP to isolated PDGFR $\alpha$ <sup>+</sup> cells also elicited hyperpolarisation via P2Y<sub>1</sub>, a purinergic G protein-coupled receptor (P2Y)<sup>62</sup>. In the gut, gap junctions are thought to mediate signal transduction between ICs and SMCs<sup>47,63</sup>. Co-localisation of the gap junction protein, connexin 43 with ICs in humans and mice<sup>52,64</sup>, supports a role for signal transduction between ICs and SMCs in the bladder. As such, gap junctions may facilitate hyperpolarisation of SMCs, potentially explaining the biphasic contractile response of SMCs to ATP, which is characterised as an initial contraction followed by sustained relaxation<sup>61,62</sup>.

Many of the functions attributed to the LP are dependent on the rich nerve plexus found here. Indeed, afferent and efferent fibres have been identified in the LP of the human bladder by transmission electron microscopy and antibody-specific immunolabelling for PGP9.5, vAChT, AChE, calcitonin gene-related peptide (CGRP), Substance P (SP), neurokinin A (NKA), neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS), vasoactive intestinal peptide (VIP), ATP-gated P2X receptor cation channels (P2X), and transient receptor potential cation channel, subfamily V, member 1 (TRPV1), among other markers<sup>39,46,49,65-70</sup>. In the rat, CGRP<sup>+</sup> nerves form an elaborate plexus just below the basement membrane<sup>71</sup>. A subset of these nerves were described as distinctly varicose and imaged running parallel to the basement membrane with branches extending into the urothelium, some of which penetrated almost the full urothelial thickness (Figure 1-1). In the mouse, almost 10% of bladder afferents are found in the urothelium<sup>72</sup>. An extensive suburothelial plexus is also well characterised in humans<sup>68,69</sup>.

Furthermore, intramural ganglion cells have been identified throughout the LP and embedded along smooth muscle bundles of the human bladder<sup>65,66</sup>. Further study using electron microscopy identified these as small in size, containing one to six neurons each<sup>66</sup>. Highlighting the complexity of bladder innervation, nerves positive for CGRP, SP, and NKA encircled the intramural ganglia and vasculature, while the VIP<sup>+</sup> nerves were found within the suburothelial plexus and projected into detrusor muscle bundles<sup>67</sup>.

#### 1.1.1.2 Detrusor muscle

The detrusor muscle provides the contractile power of the bladder through coordinated contraction and relaxation of SMCs. The detrusor is comprised of smooth muscle bundles of various sizes that contain staggered SMCs. The smooth muscle cells are long, spindle-shaped cells with a central nucleus, which when fully relaxed are several hundred micrometres long and up to 6µm wide<sup>2</sup>, and are arranged such that the wide nuclear region of one cell is aligned with the tapered end of the adjacent cell<sup>73</sup>. Adjacent cells are attached at various points through gap junctions that allow ions to flow between cells, facilitating rapid spread of the electrical signal through the muscle<sup>2,74</sup>. These smooth muscle bundles interact with both ICs and nerves and are surrounded by connective tissue rich in collagen<sup>2</sup>. The ability of these cells to undergo drastic changes in shape is a key aspect of bladder compliance.

In adults, bladder capacity ranges from 300-400mL<sup>1</sup>; this figure is different in children as bladder capacity increases throughout development and is estimated as<sup>75</sup>: capacity (mL) = [age (years) + 2] × 30. Using a spherical bladder model, a full, 400mL bladder has a circumference of approximately 30cm. If there is a residual volume of 10mL after voiding, the circumference will have been reduced to roughly 8cm<sup>1</sup>. Accommodating such a change in circumference requires a decrease in muscle length of almost 75% in a relatively short time, a unique property of smooth muscle. Notably, this also requires a drastic change in the surface area of the urothelium without loss of barrier function, as discussed above.



Detrusor contraction is initiated by an increase in intracellular  $\text{Ca}^{2+}$ , which is primarily mediated through activation of the  $\text{M}_3$  muscarinic receptor and the  $\text{P2X}_1$  purinergic receptor. In the healthy human detrusor,  $\text{M}_3$  induced contraction predominates, yet this may change under pathological conditions<sup>2,76,77</sup>. The increase in cytoplasmic  $\text{Ca}^{2+}$  concentrations has been linked to long-lasting, voltage-dependent calcium (L-type  $\text{Ca}^{2+}$ ) channels and inositol triphosphate elicited release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum. Intracellular  $\text{Ca}^{2+}$  complexes with calmodulin and this complex binds myosin light chain kinase (MLCK), activated MLCK phosphorylates the regulatory myosin light chains. This allows the interaction of myosin with actin and subsequent force generation<sup>78</sup>.

In contrast, detrusor relaxation requires a decrease in intracellular  $\text{Ca}^{2+}$  and increase in myosin light-chain phosphatase (MLCP) activity, the enzyme responsible for dephosphorylation of the myosin regulatory chain. Detrusor relaxation in humans is mediated predominantly through the  $\beta_3$ -adrenoceptors ( $\beta_3\text{-AR}$ )<sup>2,79</sup>. Activation of  $\beta_3\text{-AR}$  stimulates adenylyl cyclase, increasing cyclic adenosine monophosphate (cAMP) levels, which activates protein kinase A (PKA). Protein kinase A inhibits the formation of inositol triphosphate and has been linked to other contractile pathways, such as  $\text{Ca}^{2+}$ -independent and Rho/Rho-kinase mediated signalling. Furthermore, PKA reduces calcium influx through activation of  $\text{K}^+$ -channels and downstream effectors of cAMP stimulate large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, leading to hyperpolarisation<sup>80</sup>.

#### 1.1.1.3 Bladder Innervation

The bladder cycles between two states, bladder filling and voiding. During bladder filling, the detrusor is relaxed and the urethra and bladder neck are contracted; however, the transition to voiding results in a comparatively fast detrusor contraction and relaxation of the urethra and bladder neck. Each of these states necessitates the coordinated action of detrusor, urethral, and sphincter muscle, requiring a high level of regulation.

Three major nerves innervate the bladder, urethra, and sphincters each with afferent and efferent neurons located within the LUT. The sympathetic pathways of the hypogastric

nerve relax the bladder body while exciting the base and urethra. Parasympathetic pathways travel via the pelvic nerve; these neurons excite the bladder body and relax both the bladder neck and urethra. The pudendal nerves are associated with excitation of the external urethra through the somatic efferent pathway<sup>1</sup>. Application of axonally transported dyes highlights the cell bodies of these neurons in the lumbosacral dorsal root ganglion and terminations in the spinal cord.

Post-ganglionic parasympathetic nerves predominantly terminate in the detrusor and release ACh, while sympathetic nerves primarily terminate in the lamina propria and urothelium where they release norepinephrine (NE)<sup>81</sup>. Indeed, most nerves running in the detrusor are positive for AChE and vAChT<sup>16,65,81</sup>. Cholinergic input is supported by electrical field stimulation studies, which identify ACh and ATP as providing the bulk of the excitatory input. Notably, these efferents also stain for VIP, NPY, and nitric oxide synthase (NOS)<sup>81</sup>, suggesting the release of multiple mediators. Nerves positive for tyrosine hydroxylase may also indicate the presence of adrenergic efferents<sup>16</sup>.

Afferent nerve identification is traditionally made through morphological and molecular criteria; morphologically, two populations of afferent fibres are found in the bladder, thinly myelinated A $\delta$  and small unmyelinated C-fibres. Each of these can be further characterised based on immunolabelling. For instance, CGRP<sup>+</sup> afferents are abundant in the bladder wall, being found in the urothelium, LP, detrusor muscle and serosal blood vessels. Notably, these nerves are distinctly varicose, and these varicosities serve as release sites for stored neurotransmitters, indicating a role for afferents in “axon reflexes”<sup>70,81,82</sup>. Once activated, afferents convey information to the central nervous system and mediate localised responses through the release of molecules to proximal cells, such as SMCs, ICs, urothelial cells, and other neurons. In humans, CGRP<sup>+</sup> fibres are rarely identified in the detrusor muscle, yet are frequently found within the LP where they integrate within the suburothelial plexus. Additionally, some synapse on intramural ganglia, providing a basis for local neural reflexes<sup>67-70</sup>.

There is a differential distribution of afferents within the human bladder, wherein C-fibres are present throughout the urothelium and lamina propria, while A $\delta$ -fibres are

located in the detrusor layer<sup>53,82-84</sup>. Further, the suburothelial plexus is most dense within the bladder neck and initial part of the urethra yet becomes progressively less so towards the bladder dome. In contrast, afferent innervation of the detrusor is uniform throughout the bladder wall<sup>53</sup>. Interestingly, different peptide expression profiles exist in the neurons of detrusor and mucosa and are identified through co-localisation experiments. For example, nerves in the human bladder have been documented that are positive for CGRP and SP, NKA, or NPY<sup>67,68</sup>.

At present, there is no consensus on a functional categorisation system for afferent neurons. Some groups categorise bladder afferents as distension sensitive mechanoreceptors, distension insensitive chemoreceptors, and silent afferents<sup>85</sup>; while other groups categorise by location in the bladder wall and normal function. For example, Yoshimura and Chancellor suggest A $\delta$ -fibres of the smooth muscle sense bladder fullness (wall tension), while C-fibres of the mucosa respond to stretch (bladder volume), are nociceptive to over-distension, or silent afferents<sup>1</sup>. Silent C-fibres respond primarily to noxious stimuli, including chemical irritation and cooling<sup>76</sup>. Irrespective of the categorisation system, studies concede that nerves of the urinary bladder respond to a wide variety of stimuli. Furthermore, aberrant sensory feedback has been implicated in multiple disease states, including IC/BPS, overactive bladder (OAB), and idiopathic detrusor overactivity (IDO)<sup>2,77,81,86-88</sup>.

### 1.1.2 Micturition

As described above, an intricate neural signalling network is involved in the coordinated response of the urinary bladder and urethra in micturition<sup>2,76</sup>. Central control of the LUT is organised as a simple “on/off” switch that maintains a reciprocal relationship between the bladder and urethra. Importantly, as the bladder fills, gradual increases in bladder pressure activate sensory nerves, and this has been suggested to communicate fullness to the brain<sup>88</sup>. In humans, bladder fullness is sensed at an intravesical pressure of 5-15 mmHg, triggering an increase in bladder afferent signalling. Urgency occurs at 20-25 mmHg, and if not relieved, pain and/or discomfort are felt once the bladder exceeds 30 mmHg<sup>89</sup>. However, sensations of fullness alone do not trigger the void reflex in healthy

adults or toilet-trained children rather micturition onset is voluntary and dependent on learned behaviour.

During the storage phase, sympathetic nerves release NE, which acts on  $\beta$ 3-AR in the detrusor and  $\alpha$ -adrenoceptor ( $\alpha$ -AR) in the urethra and bladder neck, while the pudendal nerve contracts the urethra. When the bladder wall tension exceeds the micturition threshold, increased afferent firing from tension receptors in the bladder reverses the efferent outflow, inducing parasympathetic release of ACh and ATP, and inhibiting sympathetic and somatic pathways. The expulsion phase is initiated by relaxation of the urethral sphincter via nitric oxide (NO) release and the removal of adrenergic and cholinergic input. Urethral relaxation is followed by bladder contraction, which increases bladder pressure and initiates urine flow. Furthermore, urine flow through the urethra activates afferent nerves that further induce bladder contraction, promoting complete bladder emptying<sup>76</sup>.

Interestingly, the detrusor is not silent during filling; uncoordinated spontaneous activity has been documented in multiple species, including humans, and these are often referred to as non-voiding or transient contractions<sup>76,90,91</sup>. Although the function of non-voiding contractions is unknown, a number of research groups have supported their role as a sensory system<sup>47,90</sup>. Researchers argue transient contractions activate afferent nerves to send information to the brain regarding bladder fullness and the ability of the detrusor to maximally and efficiently contract, thus signalling the optimal time to void. An increase in amplitude and frequency of these contractions as the bladder fills supports this notion<sup>6,40</sup>. The generation and transduction of these contractions are also unclear, yet has been linked to ICs<sup>38,40,47,51</sup>. Notably, these transient contractions are also observed *ex vivo* in unstimulated whole bladder and isolated detrusor strips<sup>91-93</sup>. Increased incidence of micromotions has also been correlated with pathological conditions in humans and animals<sup>2,40,76,94</sup>. For instance, use of micromotion detection catheters has linked micromotions with urinary urgency and idiopathic chronic pelvic pain in women<sup>94,95</sup>.

## 1.2 Microbiome of the urinary tract

The microbiota is defined as the microorganisms in a particular environment<sup>96,97</sup>. More specifically, the term refers to the microbial taxa that are associated with an environment and are revealed using molecular techniques such as 16S ribosomal ribonucleic acid (rRNA) sequencing. Conversely, the term microbiome is less firmly defined. Some groups limit the use of microbiome to the catalogue of microbes and their genes only<sup>97</sup>, whereas our group and others prefer to refer to this as the metagenome (all genetic material of a population including plasmids). The term microbiome is used as a reference to the habitat as a whole<sup>96</sup>, thus, incorporating the biotic and abiotic factors, encompassing host and microorganism genomes and environmental conditions (Figure 1-2). The populations are composed of bacteria, archaea, viruses and fungi, which are predominantly found in the gastrointestinal tract, but also in other exposed tissues, such as the skin, upper respiratory and urogenital tracts<sup>98-100</sup>. However, other tissues that were once considered sterile, such as the brain, breast, placenta and the urinary tract, also harbour unique bacterial communities<sup>101-104</sup>.

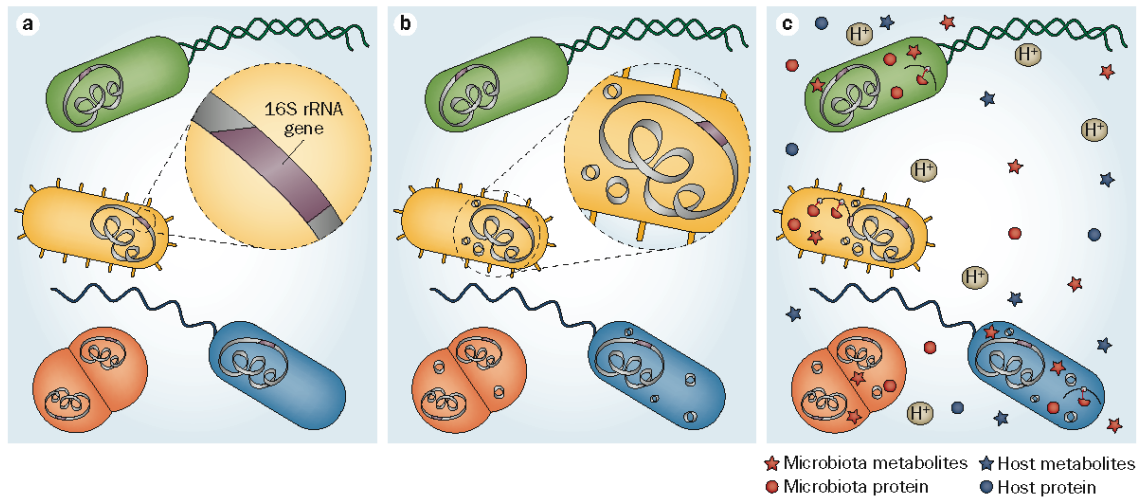
The recent identification of a microbiota in the bladder has important implications for the urologist. Studies of other body systems suggest that the microbiota is critical in the maintenance of health and/or development of disease. For example in the gastrointestinal tract, links between gut dysbiosis, chronic *Clostridium difficile* infection and colorectal cancer have been reported<sup>105-107</sup>. For the urinary tract, researchers have only begun to assess the relationship between the urinary microbiota and urologic disease.

Such studies suggest that the urinary tract harbours a unique urinary microbiota<sup>102,108-119</sup>, which is substantially different from the populations of the gut and vagina. These findings challenge the long-held doctrine of the urinary tract proximal of the urethra as a sterile environment and raise the question of why these bacteria had not been discovered previously.

### 1.2.1 Discovery of the urinary microbiota

Traditionally, bacteriological culture of urine is used to isolate and identify pathogens involved in the development of UTIs; for example, aerobic, fast-growing organisms, such as *E. coli* and *Enterococcus faecalis*<sup>120</sup>. Conversely, slow-growing, anaerobic or fastidious organisms, such as *Corynebacterium*, *Lactobacillus* and *Ureaplasma*, are rarely isolated from the urinary tract, as routine culture techniques are not designed to support the growth of these genera<sup>108,121-123</sup>. However, using more advanced deoxyribonucleic acid (DNA) sequencing-based detection technologies, these bacteria have been identified as members of the urinary microbiota in multiple studies<sup>109-113</sup>. Similarly, owing to such advanced culture and molecular techniques, *Lactobacillus iners* replaced *Lactobacillus acidophilus* as the generally accepted dominant species in the microbiota of the healthy vagina<sup>124,125</sup>. Hence, improvements in polymerase chain reaction (PCR) and 16S rRNA sequencing technology have made it possible to ascertain the bacterial communities of the body<sup>126</sup>, leading to the discovery of the microbiota of the urinary tract.

This revelation raises many questions. How can so many species, including some known to cause UTI, survive and multiply within the urinary tract without causing chronic infection? The required nutrients seem to be available, and presumably many organisms carry the necessary virulence factors for infection. Why would genera, such as *Jonquetella*, *Parvimonas*, *Proteiniphilum* and *Saccharofermentans*, previously unfamiliar to urologists, seemingly arbitrarily colonize the urinary tract of individuals aged >70 years and what are these organisms doing<sup>117</sup>?



**Figure 1-2. Definition of microbiota, metagenome, and microbiome.**

Each image represents the same population; however, different approaches to define the population provide different information. Microbiota: 16S rRNA surveys are used to taxonomically identify the microorganisms in the environment **(A)**. Metagenome: the genes and genomes of the microbiota, including plasmids, highlighting the genetic potential of the population **(B)**. Microbiome: the genes and genomes of the microbiota, as well as the products of the microbiota and the host environment **(C)**.

Stapleton<sup>127</sup> has suggested that non-modifiable host factors have a role in colonisation by these organisms. If host factors indeed ‘select’ the species that become established, what are the receptors and the basis for selection in evolutionary terms for a given individual? Did bacteria and their hosts coevolve? This process might encompass human urinary tract adaptation to accommodate certain bacterial species, for example, through expression of specific receptors, as well as mutations in bacteria enabling adherence to the uroepithelium and survival in the variable urinary conditions. Clinically, it is known that children of women with a history of recurrent UTIs often also have recurrent infections<sup>128</sup>. Is this increased susceptibility to UTIs caused by genetic inheritance or the transfer of bacterial species throughout childhood or, more specifically, at birth?

### 1.2.2 Alterations and disease

Investigations of bacterial populations in the urinary tract of healthy volunteers and patients with different diseases have revealed an altered microbiota in individuals with IC/BPS, neurogenic bladder dysfunction (NBD), urgency urinary incontinence (UUI) and asymptomatic sexually transmitted infections (Table 1-1)<sup>109,113-116</sup>. In patients with NBD, the alterations to the microbiota seem to positively correlate with the duration of the condition and the type of catheter used in bladder emptying<sup>114</sup>. The latter is intriguing and supports the ‘receptivity’ concept, whereby the presence of a new surface ‘attracts’ certain organisms, changing the dynamics of the microbiota. Urine from patients with IC/BPS had lower bacterial diversity than urine from healthy volunteers compounded by an increase in the relative abundance of the genus *Lactobacillus* (92% versus 57%)<sup>113</sup>. This finding raises two questions. First, is the loss of diversity a cause of or caused by the condition? Second, why is the increase specific for *Lactobacillus*, a genus that is considered beneficial in both the intestine and vagina, in a diseased state<sup>105,129,130</sup>? Interestingly, reduced bacterial diversity has also been correlated with other chronic inflammatory states, including obesity and inflammatory bowel disease<sup>131,132</sup>. Evidently, urinary microbiota studies are providing a whole range of novel ideas about urinary health and disease, yet still demonstrate that the microbiota of patients with symptomatic UTIs are dominated by organisms that have previously also been identified via traditional culture methods<sup>118</sup>.



### 1.2.3 Microbiota of different populations

Although the numbers of studies are limited, some have found significant differences between the urinary microbiota of men and women<sup>114,117</sup>. This finding is not surprising given the differences in anatomical structure, hormones and local defences, but it is worthy of study in relation to disease susceptibility. For example, analysis of the EURO CARE-4 cancer survival data highlighted the disparity in bladder cancer survival between the sexes: the age-adjusted 5-year relative survival was 4.2% lower for women compared with men<sup>133</sup>. By contrast, when the data from various organs was combined, the report noted a significant survival advantage for women. As an example regarding non-cancerous urinary tract diseases, one study demonstrated that men have a higher incidence of renal calculi compared with women<sup>134</sup>. It is possible that differences in the urinary microbiota of men and women have a role in these disparities.

To date, the variations in detected organisms between samples have been too high to indicate that comparable bacterial communities in individuals of the same age or gender exist<sup>112,115-117</sup>. However, Lewis *et al.*<sup>117</sup> propose the presence of a ‘core’ bladder microbiota—a subset of bacteria that exist at variable abundances within the urinary tract regardless of age. This hypothesis needs to be confirmed by large studies accompanied by investigations to identify why these organisms have evolved with the human urinary tract. This evolution could encompass coevolution, but also changes in the urinary microbiota with an individual’s age: the microbiota of children are likely to differ from adults, and the microbiota of adults are likely to differ within age groups, for example, owing to changes in urinary metabolites, personal hygiene and voiding habits.

**Table 1-1. Studies characterising the urine microbiota\*.**

Study	Participants (n)	Notable taxa <sup>†</sup>	Sample collection method
Nelson <i>et al.</i> (2010) <sup>115</sup>	Men with STI (10) Men without STI (9)	<i>Lactobacillus</i> , <i>Sneathia</i> , <i>Gemella</i> , <i>Aerococcus</i> , <i>Corynebacterium</i> , <i>Streptococcus</i> , <i>Veillonella</i> , <i>Prevotella</i> , <i>Anaerococcus</i> , <i>Propionibacterium</i> , <i>Atopobium</i> , <i>Staphylococcus</i>	First-void urine
Dong <i>et al.</i> (2011) <sup>116</sup>	Men with STI (10) Men without STI (22)	<i>Lactobacillus</i> , <i>Sneathia</i> , <i>Veillonella</i> , <i>Corynebacterium</i> , <i>Prevotella</i> , <i>Streptococcus</i> , <i>Ureaplasma</i> , <i>Mycoplasma</i> , <i>Anaerococcus</i> , <i>Atopobium</i> , <i>Aerococcus</i> , <i>Staphylococcus</i> , <i>Gemella</i> , <i>Enterococcus</i> , <i>Finegoldia</i> , <i>Neisseria</i> , <i>Propionibacterium</i> , <i>Ralstonia</i>	First-void urine
Siddiqui <i>et al.</i> (2011) <sup>112</sup>	Healthy women (8)	<i>Lactobacillus</i> , <i>Prevotella</i> , <i>Gardnerella</i> , <i>Peptoniphilus</i> , <i>Dialister</i> , <i>Finegoldia</i> , <i>Anaerococcus</i> , <i>Allisonella</i> , <i>Streptococcus</i> , <i>Staphylococcus</i>	Clean-catch midstream urine
Fouts <i>et al.</i> (2012) <sup>114</sup>	Healthy controls (26; 58% women) Patients with NBD (27; 48% women)	Orders: Lactobacillales, Enterobacteriales, Actinomycetales, Bacillales, Clostridiales, Bacteroidales, Burkholderiales, Pseudomonadales, Bifidobacteriales, Coriobacteriales	Midstream urine, intermittent catheterisation, Foley catheter
Nelson <i>et al.</i> (2012) <sup>111</sup>	Healthy adolescent men (18)	<i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Sneathia</i> , <i>Mycoplasma</i> , <i>Ureaplasma</i>	First-void urine
Siddiqui <i>et al.</i> (2012) <sup>113</sup>	Women with IC/BPS (8)	<i>Lactobacillus</i> , <i>Gardnerella</i> , <i>Corynebacterium</i> , <i>Prevotella</i> , <i>Ureaplasma</i> , <i>Enterococcus</i> , <i>Atopobium</i> , <i>Proteus</i> , <i>Cronobacter</i>	First-void urine
Wolfe <i>et al.</i> (2012) <sup>102</sup>	Healthy women (12) Women with POP or UI (11)	<i>Lactobacillus</i> , <i>Actinobaculum</i> , <i>Aerococcus</i> , <i>Anaerococcus</i> , <i>Atopobium</i> , <i>Burkholderia</i> , <i>Corynebacterium</i> , <i>Gardnerella</i> , <i>Prevotella</i> , <i>Ralstonia</i> , <i>Sneathia</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> , <i>Veillonella</i>	Clean-catch midstream urine, suprapubic aspirate, transurethral catheterisation
Lewis <i>et al.</i> (2013) <sup>135</sup>	Healthy men (6) Healthy women (10)	<i>Jonquetella</i> , <i>Parvimonas</i> , <i>Proteiniphilum</i> , <i>Saccharofermentans</i> Phyla: Actinobacteria, Bacteroidetes	Clean-catch midstream urine
Fricke <i>et al.</i> (2014) <sup>110</sup>	Patients receiving first renal transplant (60; 37% women)	<i>Lactobacillus</i> , <i>Enterococcus</i> , <i>Pseudomonas</i> , <i>Streptococcus</i> Families: Bifidobacteriaceae, Corynebacterineae	Not described

Study	Participants (n)	Notable taxa <sup>†</sup>	Sample collection method
Hilt <i>et al.</i> (2014) <sup>108</sup>	Healthy women (24) Women with OAB (41)	<i>Lactobacillus</i> , <i>Corynebacterium</i> , <i>Streptococcus</i> , <i>Actinomyces</i> , <i>Staphylococcus</i> , <i>Aerococcus</i> , <i>Gardnerella</i> , <i>Bifidobacterium</i> , <i>Actinobaculum</i>	Transurethral catheterisation
Pearce <i>et al.</i> (2014) <sup>109</sup>	Healthy women (58) Women with UIUI (60)	<i>Gardnerella</i> , <i>Lactobacillus</i> , <i>Actinobaculum</i> , <i>Actinomyces</i> , <i>Aerococcus</i> , <i>Arthrobacter</i> , <i>Corynebacterium</i> , <i>Oligella</i> , <i>Staphylococcus</i> , <i>Streptococcus</i>	Transurethral catheterisation
Willner <i>et al.</i> (2014) <sup>118</sup>	Patients with acute uncomplicated UTI (50; 76% women)	<i>Anaerococcus</i> , <i>Peptoniphilus</i> , <i>Streptococcus</i> , <i>Lactobacillus</i> , <i>Staphylococcus</i> , <i>Escherichia</i> , <i>Pseudomonas</i>	Midstream urine
Horwitz <i>et al.</i> (2015) <sup>136</sup>	Elderly patients requiring catheterisation (4; 50% women)	<i>Acinetobacter</i> , <i>Escherichia-Shigella</i> , <i>Pseudomonas</i> , <i>Staphylococcus</i> , <i>Prevotella</i> , <i>Actinobaculum</i> , <i>Lactobacillus</i> , <i>Dialister</i> , <i>Bifidobacterium</i> , <i>Corynebacterium</i>	Transurethral catheterisation
Pearce <i>et al.</i> (2015) <sup>137</sup>	Women with UIUI (90)	<i>Lactobacillus</i> , <i>Gardnerella</i> , <i>Prevotella</i> , <i>Staphylococcus</i> , <i>Aerococcus</i> , <i>Bifidobacterium</i>	Transurethral catheterisation
Nickel <i>et al.</i> (2016) <sup>†138</sup>	Men with urologic CPPS (110) Men with non-urological CPPS (115)	<i>Burkholderia</i> , <i>Propionibacterium</i> , <i>Staphylococcus</i> , <i>Streptococcus</i> , <i>Finegoldia</i> , <i>Lactobacillus</i>	First-void and midstream urine
Nickel <i>et al.</i> (2016) <sup>†139</sup>	Women with urologic CPPS (213)	<i>Bifidobacterium</i> , <i>Finegoldia</i> , <i>Lactobacillus</i> , <i>Propionibacterium</i> , <i>Staphylococcus</i> , <i>Streptococcus</i>	First-void and midstream urine
Ollberding <i>et al.</i> (2016) <sup>140</sup>	Women in second trimester of pregnancy (97)	<i>Lactobacillus</i> , <i>Serratia</i> , <i>Prevotella</i> , <i>Atopobium</i> , <i>Gordonia</i> , <i>Sutterella</i> , <i>Streptococcus</i> , <i>Shuttleworthia</i> , <i>Kocuria</i> , <i>Blautia</i> , <i>Ruminococcus</i>	Not described
Shoskes <i>et al.</i> (2016) <sup>141</sup>	Men with CPPS (25) Healthy controls (25)	<i>Bacteroides</i> , <i>Blautia</i> , <i>Faecalibacterium</i> , <i>Ruminococcus</i> , <i>Coproccoccus</i> , <i>Staphylococcus</i> , <i>Corynebacterium</i> , <i>Porphyromonas</i> Order: Clostridia	Midstream urine
Thomas-White <i>et al.</i> (2016) <sup>142</sup>	Women with UIUI (37) Healthy controls (26)	<i>Staphylococcus</i> , <i>Enterococcus</i> , <i>Atopobium</i> , <i>Lactobacillus</i> , <i>Gardnerella</i> , <i>Streptococcus</i> , <i>Aerococcus</i> , <i>Actinomyces</i> , <i>Corynebacterium</i>	Transurethral catheterisation

Study	Participants (n)	Notable taxa <sup>†</sup>	Sample collection method
Abernethy <i>et al.</i> (2017) <sup>143</sup>	Women with IC (20) Healthy controls (20)	<i>Lactobacillus</i>	Transurethral catheterisation
Liu <i>et al.</i> (2017) <sup>144</sup>	Women with T2DM (70) Healthy controls (70)	<i>Prevotella</i> , <i>Lactobacillus</i> , <i>Shuttleworthia</i> , <i>Streptococcus</i> , <i>Acinetobacter</i> , <i>Bacteroides</i> , <i>Halomonas</i> , <i>Blautia</i> , <i>Faecalibacterium</i> , <i>Corynebacterium</i> , <i>Klebsiella</i> , <i>Pseudomonas</i> , <i>Coprococcus</i> , <i>Phascolarctobacterium</i> , <i>Dorea</i> , <i>Shewanella</i>	Modified midstream urine
Modena <i>et al.</i> (2017) <sup>145</sup>	Renal transplant patients with (25; 48% women) and without (23; 26% women) IFTA Healthy controls (20; 50% women)	<i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Gardnerella</i> , <i>Prevotella</i> , <i>Corynebacterium</i>	Clean-catch midstream urine
Rani <i>et al.</i> (2017) <sup>146</sup>	Renal transplant patients (21; 33% women) Healthy controls (8; 63% women)	<i>Enterococcus</i> , <i>Escherichia</i> , <i>Propionibacterium</i> , <i>Corynebacterium</i> , <i>Mobiluncus</i>	Not described
Thomas-White <i>et al.</i> (2017) <sup>147</sup>	Women with SUI (197)	<i>Lactobacillus</i> , <i>Streptococcus</i> , <i>Bifidobacterium</i> , <i>Gardnerella</i> , <i>Corynebacterium</i> , <i>Atopobium</i> , <i>Prevotella</i>	Not described

\*Included studies characterise the microbiota through use of next-generation sequencing technology unless otherwise noted.

<sup>†</sup>Identified by authors of the original study as predominant or as significantly more prevalent than other populations; listed as genera, unless otherwise noted.

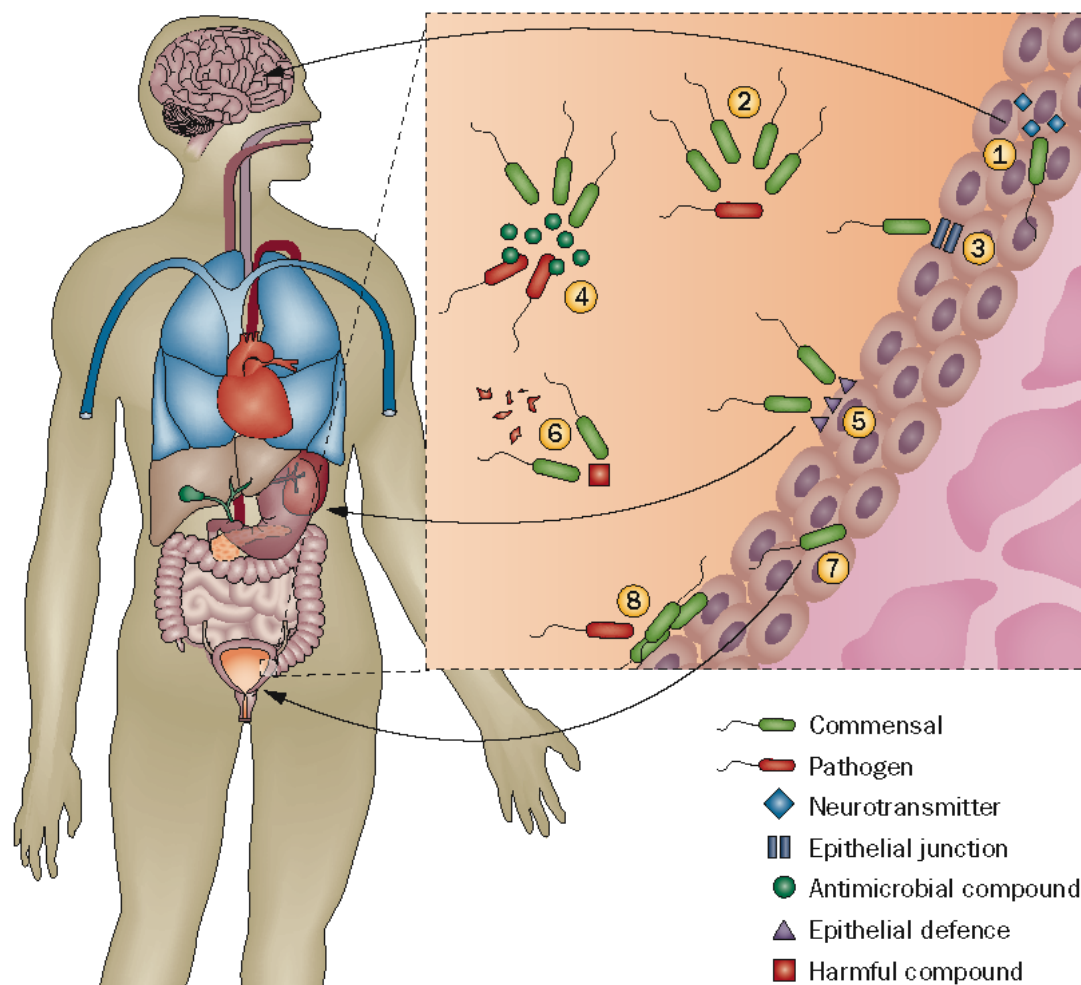
\*Microbiota analysis completed using the Ibis T-5000 Universal Biosensor system.

Abbreviations: CPPS, chronic pelvic pain syndrome; IC/BPS, interstitial cystitis/bladder pain syndrome; IFTA, interstitial fibrosis and tubular atrophy; NBD, neurogenic bladder dysfunction; POP, pelvic organ prolapse; STI, sexually transmitted infection; SUI, stress urinary incontinence; T2DM, type 2 diabetes mellitus; UI, urinary incontinence; UTI, urinary tract infection; UUI, urgency urinary incontinence.

### 1.2.4 Maintenance of homeostasis

Assuming a mutualistic relationship, in which the bacteria benefit from the host's nutrient supply, pH, oxygen concentration and other survival factors, it remains unclear what advantage the microbiota provide to the host. The human microbiome is critical in the maintenance of health and development at different sites throughout the body, but whether the urinary microbiota have a role similar to that of bacterial communities at other mucosal sites requires further investigation (Figure 1-3). In addition, it is known that microbes are important in establishing the immune system after birth and maintaining its effectiveness throughout life<sup>148</sup>. The presence of dedicated immune cells within the urinary tract raises the question of whether the urinary microbiota has a similar role in priming the immune system.

Bacteria are also able to interact with many environmental toxins, such as heavy metals, polycyclic aromatic hydrocarbons, pesticides, ochratoxins, plastic monomers, and organic compounds<sup>149-152</sup>. After certain toxins have been removed from the blood stream through renal filtration, their storage within the bladder provides ample time for the urinary microbiota to interact with and alter these compounds. This 'metabolism' can increase or decrease the risk of diseases, including cognitive dysfunction, renal pathologies and urinary cancers, that can be caused by these toxins<sup>153</sup>.



**Figure 1-3. Potential roles of the urinary microbiota in homeostasis.**

Bacteria might produce neurotransmitters that interact with the nervous system (1). Commensal bacteria might outcompete pathogens for common resources (2). Bacteria might have a role in the regulation and maintenance of epithelial junctions (3). Commensals might produce antimicrobial compounds that kill pathogens (4). Bacteria might prime epithelial defences, including immune defences (5). Commensal bacteria might degrade harmful compounds (6). Bacteria might be necessary for proper development of the urinary tract, including the uroepithelium, immune system, and peripheral nervous system within the bladder and surrounding tissues (7). Commensals might create a barrier, blocking pathogen access to the uroepithelium (8).

The gut microbiota has been linked to the development of both the enteric and central nervous system after birth<sup>154</sup>. However, little is known about bacterial interactions with the peripheral nervous system. The urinary microbiota might be required for correct development of and signalling within this system, potentially through the production of neurotransmitters or interactions with these systems. Loss of these functions might be the cause of diseases, such as OAB and IC/BPS. Indeed, studies on germ-free mice show that the absence of microbes correlates with a compromised immune system, leaky gut, as well as behavioural and neurological disorders<sup>155</sup>. Studies of urinary tract function in germ-free animals are rare, so it is currently unclear exactly how this system is influenced by the microbiota. Finally, the urinary microbiota might act as a barrier to uropathogens, for example, through competition for resources, similar to other body sites<sup>156</sup>. In the vagina, secretions can have inhibitory activity against *E. coli*, particularly in communities dominated by *Lactobacillus crispatus*<sup>157</sup>.

One of the limitations of 16S rRNA sequencing is its inability to differentiate between live and dead bacteria or bacterial DNA fragments. Hilt *et al.*<sup>108</sup> addressed this issue by expanding culture conditions beyond the ones that are used routinely to isolate bacteria from urine. Using these modified culture criteria, 80% of 65 samples, of which 92% were culture negative (<10<sup>3</sup> colony forming units (CFU)/mL) using traditional culture techniques, grew bacteria. Comparison of these findings to their high-throughput sequencing results suggested that a majority of the bacteria found within the urinary microbiota via 16s rRNA sequencing were indeed alive.

### 1.2.5 Factors influencing the microbiota

It is well known that dietary factors can influence the risk of urinary infections and calculi. Consistent high water intake is important, but it is perhaps a little simplistic to suggest that it can help cure UTIs. Indeed, urine from individuals with increased water intake significantly increased the initial deposition rates and numbers of adherent *E. coli* and *E. faecalis* to silicone rubber<sup>158</sup>. This finding is of particular importance in patients with catheter-associated UTI (CAUTI) and suggests that high water intake leads to the dilution of a urinary factor that inhibits microbial deposition, rendering it ineffective.

Notably, a proportion of UTI cases resolve without intervention for unexplained reasons<sup>159-161</sup>.

These findings raise a number of questions. Does the urinary microbiota simply need time to reconstitute following an acute infection? If so, why do not all infections resolve without intervention? Are there other triggers of infection, for example, alterations in the gut microbiota? Many microbial metabolites are found in the urine, which can be used as an indicator of gut dysbiosis. For example, in rats, in which the microbiota was suppressed by antibiotic treatment, the urinary metabolite profiles between the treated and control rats were remarkably different<sup>162</sup>. Clearly, many waste products, including microbial metabolites, are excreted via the urinary tract, but whether these metabolites are harmful to the urinary microbiota and their role in the development of UTIs is unclear. Multiple groups have explored the use of nuclear magnetic resonance (NMR) spectroscopy to differentiate the metabolites in the urine from healthy patients and those with suspected UTI<sup>163</sup>, as well as to identify biomarkers of morbidity<sup>164</sup>.

Consumption of cranberry juice has been proposed to reduce the incidence of recurrent UTIs; however, in 2012, a randomised controlled trial in women with a history of recurrent UTI found no protective effects<sup>165</sup>. Other studies have suggested that D-mannose, a component of cranberry juice and other juices, especially pineapple juice, inhibits the attachment of bacterial type 1 fimbriae to cell surfaces, presumably reducing the pathogen's ability to remain in the urinary tract and infect the host<sup>166</sup>. Although more studies are needed—which should also examine the effect on the total microbiota—urinary components indeed seem likely to have an important role in determining which organisms inhabit the urinary tract.

In addition, the virome is likely to be important in maintaining the urinary microbiota. The virome is defined as the set of viruses infecting eukaryotic, bacterial, and archaic microorganisms found within the host, as well as the virus-derived genetic elements that have integrated chromosomally<sup>167</sup>. Similar to the bacterial contingent of the microbiome, the virome can be found throughout the entire human body, including the urinary tract, and is not limited to the mucosal sites. To date, little is known about the influence of the



virome on the urinary microbiota and this topic is likely to receive much attention in the coming years. However, as viruses are either DNA or RNA encoded and owing to high genomic diversity, characterisation of the virome is complicated by the limitations of current technology and the lack of a universal phylogenetic marker, such as the 16S rRNA in bacteria<sup>168</sup>.

Importantly, the ways in which antibiotics and antimicrobial agents prescribed to patients, as well as trace amounts found in drinking water and food, affect the human microbiome are only now being realized<sup>169</sup>. The fact that bacterial resistance to antibiotics is increasing is well known; however, the use of low-dose, empiric prophylactic antibiotics to prevent recurrent infection remains an accepted therapeutic option. These practices might be creating bacterial persister cells that are genetically homogenous to previous generations, but exhibit a greater fitness, enabling them to be more capable of invading the uroepithelium and acting as a source of sepsis and infection, but potentially also of becoming part of the urinary microbiota<sup>170</sup>. In children with anatomical anomalies that predispose to recurrent UTI, infection is often managed with low-dose antibiotic prophylaxis—the development of persister cells due to such medication might be contributing to the recurrent nature of UTI.

Changes in bacterial diversity over the course of an individual's life have been described for the intestinal microbiota and are also likely to occur to some extent in the urinary microbiota<sup>171</sup>, given the influence of the former on the latter. Hormonal changes associated with puberty and menopause<sup>172</sup>, as well as constipation and urinary incontinence, are known to influence the microbiome<sup>173</sup>. In women, such changes primarily affect the composition of lactobacilli populations in the vagina<sup>174</sup>. In addition, in women, sexual activity can alter the microbiota of both the urethra and the vagina<sup>99</sup>. Furthermore, in male adolescents (aged 14–17 years) who have engaged in vaginal sexual intercourse, bacteria commonly associated with bacterial vaginosis can colonize the coronal sulcus and distal urethra<sup>111</sup>. These genera were not found in sexually inexperienced individuals, suggesting that colonisation was related to sexual activity. The same study also found significant differences in the types of bacteria colonizing the coronal sulcus between circumcised and uncircumcised adolescents. After puberty, the

prostate has developed sufficiently to produce prostatic fluid that contains various antimicrobial substances<sup>175</sup>, which are capable of influencing the urinary microbiota. In addition, spermicidal agents might disrupt both the male and female urinary microbiota, similar to disruptions observed in the vagina<sup>176,177</sup>. Furthermore, anal and oral penetration during sexual intercourse exposes men to two atypical microbial environments.

As has been proposed for the gut<sup>178</sup>, the balance between certain microbial groups or organisms that contribute to a conserved metagenome might be more important than the overall composition of the urinary microbiota. For example, two populations might contain completely different sets of bacterial species, yet perform the same function, as the genetic potential is present in both populations. This property adds an aspect of redundancy to the microbiome, acting as a safeguard in the maintenance of health and development of disease. It is important to note that the metagenome represents only the genetic potential of the microbiota, not the actual activity of the population.

## 1.3 Infectious states of the urinary tract

### 1.3.1 Urinary tract infections

The ongoing characterisation of the urinary microbiota has urged some researchers to suggest the traditional definition of UTI requires an overhaul. The recent urinary microbiota findings have led to the conclusion that a subset of urinary conditions may need to be reclassified as dysbiosis, rather than invasion by a single pathogen<sup>179</sup>. Such a change in dogma would elicit significant changes in clinical strategies.

The term “urinary tract infection” is widely used both within and without the clinical setting. However, the term itself is quite broad, encompassing an infection within any part of the urinary tract, such as an infection of the kidney (pyelonephritis), urethra, or ureter. The most common UTI is bacterial cystitis, a bacterial infection of the bladder; thus UTI and bacterial cystitis will be used interchangeably throughout this thesis.

Urinary tract infections are commonly classified as either complicated or uncomplicated based on patient characteristics; such categorisation is used clinically to aid in treatment determination. In general, an acute uncomplicated UTI is the acute onset of symptoms in

a healthy, pre-menopausal, non-pregnant woman without known functional or anatomical abnormalities of the urinary tract<sup>180</sup>. Complicating factors include; pregnancy, immune suppression, renal failure or transplant, male gender, healthcare-associated infection, recent hospitalisation or antibiotic exposure, and infection with a multi-drug resistant bacterium<sup>181</sup>.

The criteria involved in the diagnosis of UTI are consistently evolving, yet generally includes a combination of clinical features and the presence of bacteria in the urine. Of note, some researchers suggest UTIs can be reliably diagnosed based on symptoms alone and call for an end to laboratory culture for acute uncomplicated UTI<sup>180</sup>; unsurprisingly, these groups also argue for immediate empirical treatment. Meanwhile, other groups suggest the use of culture results is imperative and should be involved in the treatment decision-making process. Indeed, acute uncomplicated UTI in adult women is not life-threatening, and the argument has been made to delay treatment in this population<sup>182</sup>. Arguments for the immediate treatment of cystitis often cite the risk for development of increasingly severe infections, such as pyelonephritis and bacteraemia. Although this progression is relatively rare, approximately 15% of nosocomial bacteraemia cases implicate the urinary tract as the portal of entry<sup>183</sup>.

Diagnosis of acute uncomplicated UTI can be made for adult women with a high probability based on the sudden onset of dysuria, urgency, frequency, and suprapubic pain in the absence of vaginal discharge or irritation. The presence of fever and/or flank pain is highly suggestive of pyelonephritis<sup>184</sup>. Conversely, symptoms in early childhood are systemic rather than localised and often characterised by fever, lethargy, anorexia, and vomiting<sup>184-186</sup>. Laboratory diagnosis of UTI is based on the presence of significant bacteriuria, yet the cutoffs for this are widely disputed, have not been formally validated in children, and do not provide a measure of disease severity or renal involvement<sup>186</sup>. Throughout the scientific literature and clinical guidelines, the most commonly used definition of significant bacteriuria is greater than or equal to  $10^5$  CFU/mL<sup>186-188</sup>. In comparison, the European Association of Urology suggests a cutoff of  $10^4$  CFU/ml for men and Price *et al.* have taken it a step further, proposing each pathogen has a unique threshold for UTI diagnosis<sup>184,189</sup>. Additional urinary markers of UTI include the

presence of pyuria, nitrites, leukocyte esterase, protein, and blood<sup>184,186</sup>. Some research groups have also suggested urinary interleukin-6 (IL-6) and interleukin-8 (IL-8) have prognostic value for paediatric UTI and act as markers of disease severity<sup>190,191</sup>. Indeed, *in vitro* studies have demonstrated the production and release of cytokines by urothelial cells in response to whole bacteria and purified bacterial toxins, such as pyocyanin or lipopolysaccharide (LPS)<sup>192-194</sup>.

It is important to note, that traditional bacterial culture strategies used for urine in the clinical lab are focused on the identification of “classic” uropathogens, which are aerobic, fast-growing bacteria. However, in recent years a number of bacteria have been associated with UTIs that are not cultured using these traditional techniques, including *Prevotella* and *Porphyromonas*<sup>195</sup>. Microbiota analysis also highlights the presence of anaerobic and fastidious bacteria throughout the urinary tract, which has led to the development of expanded quantitative urine culture (EQUC)<sup>108,189</sup>. In brief, EQUC builds on standard microbiological urine culture practices, which involve aerobic incubation at 35°C for 24-hrs, to include incubation under high CO<sub>2</sub> and anaerobic atmospheres at 30°C and 35°C for 48-hrs.

Urinary tract infections are the second most common cause of community and hospital-acquired infection in both children and adults<sup>183,196,197</sup>, costing the American economy approximately 2.5 billion dollars annually<sup>198,199</sup>. In children, reports suggest up to 8% of children will experience a UTI before the age of 7<sup>186,200,201</sup>. While most of these studies are older, a retrospective analysis of data from a cohort of almost 75,000 children between 2001 to 2006 by Conway *et al.*<sup>200</sup>, supports these estimates, with a 4.2% cumulative incidence of UTI in children up to 6 years of age. The same study documented an increased risk for UTI in children aged 3 to 5, which had previously been suggested by a group in the Netherlands<sup>202</sup>. The authors suggest this age discrepancy could be due to dysfunctional elimination, a known risk factor for UTI<sup>186</sup>. In adults, 50-60% of women will experience at least one UTI over the course of their life<sup>203</sup>. Incidence is estimated to be particularly high in sexually active women aged 18-24, wherein one of five will report an uncomplicated UTI annually in the US<sup>204</sup>.

In all populations, UTI is causative of a decreased quality of life, associated with psychological distress, and loss of “functional” hours<sup>205</sup>. In adult women, UTI has also been correlated with dyspareunia, vulvodynia and lower urinary tract symptoms (LUTS), having a significant impact on sexual activity<sup>206</sup>. Economic loss is also notable during UTI, with women experiencing an average of 6.1 days of symptoms, 2.4 days of decreased activity, and 0.4 days of bed rest per symptomatic episode<sup>198</sup>.

It is understandable then that researchers would attempt to identify risk factors of UTI in hopes of preventing disease and/or limiting severity. Gender appears to play the largest role in UTI risk; after all, females are significantly more likely to develop a UTI than males. Infection history is also a key factor in determination of UTI risk, notably women with a history of RUTI and those with a first UTI before 15 years of age have increased risk<sup>28</sup>. Most other key risk factors are directly associated with manipulation of the genitourinary tract including; sexual activity, which induces retrograde force up the urethra, the use of contraceptive devices and spermicides that alter the vaginal and periurethral bacterial communities, urological surgery, and indwelling or intermittent catheterisation<sup>22</sup>. The duration of catheterisation is the most important risk factor for the development of CAUTI; in fact, the incidence of bacteriuria associated with an indwelling catheter is 3-8% per day<sup>207</sup>. Similarly, risk of UTI is increased by anatomical or functional defects that compromise normal urination, such as urinary obstruction, neurogenic bladder, urinary incontinence, labial adhesions, and VUR<sup>184,186,208</sup>. Diabetes mellitus, immunosuppression, chronic constipation, obesity, renal insufficiency, bacterial vaginosis, lupus erythematosus, and rheumatoid arthritis also predispose a person to UTI and RUTI<sup>184,209</sup>. Although not completely understood, genetics and advanced age have been correlated with increased risk of UTI<sup>21</sup>.

Further, increasing voiding frequency and water intake are strategies associated with RUTI treatment, which is interesting as increased voiding frequency was associated with lower UTI incidence in an interventional study of clean room workers<sup>210</sup>. This could be due to a number of factors, including the correlative increase in fluid intake; however, it may also be a reflection of damage due to repeated and prolonged bladder distension<sup>211,212</sup>.

### 1.3.1.1 Aetiology

Although the exact numbers vary, *E. coli* is consistently recognised as the most common cause of UTI and is associated with more than 80% of both community and hospital-acquired infections<sup>204</sup>. After *E. coli* there is no true consensus on the most common etiological agent; however, *Enterococcus* sp., *Klebsiella* sp., *Staphylococcus* sp., *Proteus* sp., *Enterobacter* sp., *Pseudomonas* sp., *Serratia* sp., Group A and B streptococci, and *Candida* sp. are all recognised as major causes of disease<sup>21,184,196,200,213,214</sup>. With that said, case reports and clinical studies have begun to identify other agents of UTI; these commonly represent fastidious bacteria or those that are difficult to differentiate from “every day” uropathogens.

As an example, *Aerococcus urinae* is reported most often in association with elderly patients, yet a series of recent case studies have also linked this bacterium to paediatric male patients<sup>215</sup>. Identification of this bacterium is complicated as it displays similar colony morphology to  $\alpha$ -hemolytic streptococci, Gram staining properties with staphylococci and antimicrobial resistance patterns to enterococci; thus the clinical impact of *A. urinae* has likely been underestimated<sup>216</sup>. The introduction of 16S rRNA gene sequencing and matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) to clinical microbiology labs will likely correspond with an increase in the perceived prevalence of bacteria such as *A. urinae* as clinical pathogens.

Interestingly, one published study has assessed the urinary microbiota in patients with acute UTI<sup>118</sup>, which was defined as a minimum of  $10^3$  CFU/mL and a white blood cell count of greater than  $10^4$  cells/mL from midstream urine. Unsurprisingly, *E. coli* was the most common bacterial isolate and concordantly the most abundant genus identified in microbiota analysis. However, 16S rRNA sequencing identified other bacterial genera in the *E. coli*-dominated UTI samples, which may also play a role in disease. Of note, *Enterococcus* sp., were present in low abundance in almost a third of the *E. coli*-dominated samples. Enterococci do not appear to have been co-cultured with these samples, which may be due to low bacterial abundance or an indication of sample contamination, yet the potential exists for bacterial interplay between these two

uropathogens. This research also highlights the presence of anaerobic and fastidious bacteria at high abundance as determined via 16S rRNA sequencing in some active UTIs, which are not isolated using routine methods, yet may also contribute to pathogenesis, highlighting the need for EQUIC testing. Notably, the limited diversity in these samples suggests that every genus present may play a key role in regulating the transition between health and disease, or disease progression.

### 1.3.2 Recurrent urinary tract infections

Defined as three or more symptomatic UTIs in a 12-month period or two or more within six-months, RUTI is one of the greatest challenges associated with acute uncomplicated UTI. Recurrence rates are high in UTI patients, although reports vary on the exact incidence rate. In one study, 25% of women with acute UTI experienced recurrence within 6 months<sup>205</sup>; another suggests 25% recurred within 12-months and 22% went on to develop RUTI<sup>217</sup>. A more conservative estimate suggests up to 5% of women experiencing their first UTI will go on to develop three or more per year<sup>205</sup>. Global estimates suggest 1% of women will experience more than six UTIs per year<sup>218</sup>. In the United States, almost 11% of women over 18 will report at least one UTI over 12 months, with the majority of these cases in women with a history of RUTI<sup>219</sup>. Aetiology and risk factors are similar to those of UTI, with increased weight placed on a previous history of UTI, index infection at an early age, and maternal history of UTI<sup>184</sup>.

In children, a retrospective study of almost 75,000 children demonstrated an incidence of recurrence of 12% per year in a primary care setting<sup>200</sup>. In contrast, studies on referral and emergency department populations estimate paediatric recurrence rates at 20-48% in the first 6-12 months<sup>185,220</sup>. Risk factors for paediatric RUTI include poor hygiene and voiding habits, as well as constipation and index infection before 12-months of age. In fact, recurrence rates in children with a UTI before one year are 75%, and this drops to 40% in girls and 30% in boys at the one year mark<sup>186</sup>.

Pathogenesis of RUTI is not well understood, although studies of *E. coli* RUTI suggest recurrent infections are often caused by the same strain. Recurrence may be a result of relapse, wherein infection occurs within 14 days of the previous and is related to

incomplete clearance; or may be due to reinfection from external sites, such as the gastrointestinal tract or vagina. Work by Scott Hultgren's group has shown UPEC invade host tissues and form intracellular bacterial communities (IBCs) in the murine bladder, these communities are associated with latent infection, yet extrusion provides an additional explanation for RUTI<sup>221</sup>. Furthermore, exfoliated IBCs have been identified in urine samples from women with UTI, supporting the applicability of this theory to human RUTI<sup>222</sup>. Following this model of latent RUTI in mice, Gilbert *et al.*, triggered *E. coli* emergence into the urine through transient exposure to *Gardnerella vaginalis*<sup>218</sup>. This led the researchers to propose a new paradigm for RUTI, covert pathogenesis. In brief, the classical view of UTI diagnosis and treatment is based on the pathogen present at the time of clinical pathogenesis, in this case, *E. coli*. Covert pathogenesis, conversely, suggests transient exposure to *G. vaginalis* triggered *E. coli* to cause disease, despite *G. vaginalis* being absent at the time of disease emergence. The theory suggests that long-term exposure is not necessary, merely a transient one and that this is likely missed in the clinical setting as they occur before symptom onset, but may drive recurrence and disease severity caused by a recognised pathogen. If this holds true in humans, it may explain the link between RUTI and sexual activity and would not necessarily apply to only *G. vaginalis* and *E. coli*. Demonstration of covert pathogenesis in humans would also support the reclassification of UTI to dysbiosis.

Recurrence may also be aided by pathogen instigated remodelling of the bladder mucosa as has been demonstrated by Scott Hultgren's group (section 1.1.1.1.1)<sup>28</sup>. In this model a bimodal outcome is associated with chronic murine bladder infection; spontaneous resolution or antibiotic-induced sensitisation. Interestingly, this pattern mimics the spontaneous resolution of both symptoms and bacteriology noted in 25%-75% of women with UTI<sup>204,223</sup>. Sensitised mice exhibit altered bladder pathology, similar to the urothelial hyperplasia and defects in terminal differentiation noted in females with a history of UTI<sup>34,224</sup>. Further, on subsequent challenge, sensitised mice experience a significantly more severe UTI compared to naïve mice. Thus, UTI may induce lasting changes to the bladder's barrier, which increases host sensitivity to infection, partially explaining previous infection as a risk factor for RUTI.



### 1.3.3 Asymptomatic bacteriuria

At the other end of the spectrum is asymptomatic bacteriuria (ABU), which is classified as the presence of significant bacteriuria without the signs or symptoms of UTI<sup>188</sup>.

Asymptomatic bacteriuria is a fairly common condition, occurring in 3-8% of premenopausal and 4-43% of postmenopausal women<sup>225</sup>. The aetiology of ABU is much the same as UTI and RUTI; *E. coli* is implicated most often, followed by *Enterococcus* sp., *Pseudomonas* sp., *Proteus* sp., *Staphylococcus* sp., and *Klebsiella pneumoniae*<sup>199</sup>. However, the bacterial cause of ABU is strongly influenced by gender, age, medical interventions (catheterisation), and comorbidities (pregnancy, diabetes, etc.). Incidence estimates suggest up to 10% of women will be diagnosed with ABU throughout their lifetime, with the state being more common in diabetics and the elderly<sup>199</sup>. Although not well understood, most studies suggest ABU is harmless in those without risk factors, such as pregnancy where ABU has been linked to preterm labour and delivery<sup>184,188</sup>. The presence of ABU does not lead to renal damage or impaired renal growth when left untreated<sup>185,226,227</sup>, and a 24-year follow-up study, demonstrated ABU was not associated with an increase in mortality or kidney disease compared to controls<sup>228</sup>.

When ABU was first identified, clinicians believed it to be harmful and worthy of eradication; with further study, this belief changed to a benign observation requiring no therapeutic intervention, unless pregnant. Indeed, multiple studies have shown antibiotic treatment of ABU to increase the risk of symptomatic UTI<sup>226,229,230</sup>; thus ABU is now interpreted as a protective state in some populations<sup>231,232</sup>. Such a protective effect has been linked to bacterial interference, wherein the ABU strain outcompetes the pathogen for nutrients or produces toxic molecules<sup>190,233</sup>.

### 1.3.4 Infection management strategies

Antibiotic administration is the standard of care for both UTI and RUTI, in fact, uncomplicated UTI is one of the most common indications for antimicrobial prescription in otherwise healthy women<sup>234</sup>. Indeed, between 2007 and 2009, UTIs were the third most likely reason for adult antibiotic prescription in the ambulatory setting, accounting for 9% of antibiotic use, 70% of which were broad-spectrum<sup>235</sup>. At present, most primary

care guidelines recommend antibiotics for UTI treatment as this provides effective and fast symptom resolution.

Current first-line antibiotics depend on the country and antibiotic resistance trends of a specific area. In the US, trimethoprim-sulfamethoxazole (TMP/SMX) is commonly used as a first line strategy<sup>234</sup>. Strong evidence also exists for the application of nitrofurantoin to the treatment of uncomplicated UTI, and indeed it is listed within the international clinical guidelines as a first-line therapy<sup>234</sup>. Interestingly, nitrofurantoin was initially approved in 1953 by the FDA for treatment of bacterial cystitis, yet fell out of style in the 1970's to TMP/SMX and the beta-lactams; however, increasing resistance to these antibiotics have led clinical guidelines to reposition nitrofurantoin as a first-line therapy<sup>236,237</sup>. In fact, resistance has been linked to relapse in 5-10% of women following completion of antimicrobial therapy<sup>225</sup>.

Canadian guidelines are no exception to these; the Toronto Central Local Health Integration Network suggests empirical treatment for uncomplicated UTI in adults using 5-days of oral nitrofurantoin as first-line followed by TMP/SMX and ciprofloxacin as second- and third-line, respectively. However, in complicated UTI TMP/SMX and ciprofloxacin make up first-line strategies<sup>238</sup>. In January 2017, the Canadian Paediatric Society (CPS) reaffirmed their guidelines for UTI in children<sup>239</sup>. In brief, CPS supports empirical treatment for UTI, using a two to four-day course of oral antibiotics, such as cefixime, TMP/SMX, amoxicillin, amoxicillin-clavulanic acid, or cephalexin. In children with febrile UTI, the course is extended to 7-10 days.

#### 1.3.4.1 Antibiotic prophylaxis for RUTI

The standard of care for prevention of RUTI is also antibiotics; yet in this case, the recommendation is long-term low dose therapy. Less common prophylactic options include post-coital antibiotics in patients where RUTI is correlated with sexual intercourse and acute self-treatment in compliant patients with a clearly documented RUTI history<sup>184,240</sup>. Trimethoprim-sulfamethoxazole daily for six months is the most recommended strategy in adults and children<sup>186,240</sup>; however, other antibiotics are also commonly used including cephalexin, amoxicillin-clavulanic acid, cefadroxil, and

nitrofurantoin<sup>184</sup>. Given that the optimal duration of antibiotic prophylaxis is unknown, many clinical guidelines suggest continued evaluation of the patient over the course of prophylactic therapy with the potential to discontinue after 3-6 months. However, 50% of women will recur within three months of discontinuation, requiring the patient “reinstitute” antibiotic therapy<sup>225</sup>.

The effectiveness of antibiotic prophylaxis for the prevention of RUTI is a contentious topic that many studies and meta-analyses have sought to address. In children, the impetus for antibiotics to treat RUTI is primarily to reduce the risk of pyelonephritis induced kidney damage<sup>185</sup>; yet, multiple studies have addressed this outcome, and none have identified a benefit to antimicrobials in the prevention of renal scarring<sup>186,220</sup>. Given the status of antibiotic prophylaxis as the standard of care, studies are lacking that have appropriately studied the effectiveness of prophylaxis, particularly in the paediatric population.

The prevention of recurrent urinary tract infection in children with VUR and normal renal tracts (PRIVENT) study<sup>220</sup>, was designed to address this question. Study coordinators recruited over 500 children who had experienced at least one culture-proven UTI; patients were asymptomatic at recruitment and during the run-in period. In this double-blind study, over 500 children who had experienced at least one culture-proven UTI were randomised to receive either placebo or TMP/SMX for 12 months. Patients were asymptomatic at recruitment and during the run-in period. Notably, and this was not touched on in their commentary, all patients received a 14-day course of TMP/SMX during the run-in period. Additionally, 15% of patients recruited in both groups had previous infections that were resistant to the antibiotic. The researchers found a small reduction in the absolute risk of UTI in predisposed children receiving probiotic (6 percentage points), meaning 14 children need to be treated with TMP/SMX for 12-months to prevent a single UTI. Half of the UTI events in the placebo group occurred in the first three months after randomisation, this is of particular interest, as this is within three months of antibiotic usage, suggesting they are not a true control group.

A potential explanation for the recurrence of infections in the placebo group lies within the treatment of ABU. As stated, none of these patients was experiencing a symptomatic UTI at recruitment, or during the run-in; however, the authors did not assess the patients for ABU. It is often noted in clinical practice that women with a history of RUTI present as symptom-free with “sterile” urine immediately after antibiotic treatment followed by ABU<sup>232</sup>. Thus, these children may have had ABU, which the run-in antibiotics would likely have disrupted. This is important to note, as antibiotic treatment of ABU is associated with poor outcomes<sup>217,226,227,229,230</sup>. In their study, Cai *et al.* noted UTI recurrence in patients treated for ABU with a history of UTI compared to those left untreated. By six months, the difference was statistically significant (7.6% vs 29.7%), yet was even more dramatic at 12 months, wherein 14.7% of the untreated patients experienced UTI compared to 73.1% of treated patients<sup>232</sup>.

Another study, randomised intervention for children with VUR (RIVUR)<sup>241</sup>, focused on patients with VUR diagnosed after a first or second febrile UTI. The results from this randomised, double-blind, placebo-controlled trial of TMP/SMX suggest prophylaxis is beneficial for prevention of RUTI in patients with VUR. However, antibiotic resistance rates in uropathogens were significantly increased in the prophylaxis group (68%) compared to the placebo group (24%). A Cochrane review came to similar conclusions; antibiotic prophylaxis reduced the risk of significant bacteriuria relative to placebo and offered a slight benefit in preventing symptomatic UTI in children while increasing the likelihood of antibiotic resistance<sup>185</sup>. In contrast, another meta-analysis did not find a significant reduction in UTI incidence associated with prophylaxis, which was supported by subgroup analyses of participants with and without VUR<sup>242</sup>. Similarly, in a retrospective study of paediatric patients receiving TMP/SMX or, when culture history indicated resistance to this antibiotic, nitrofurantoin, every child in the study had a documented UTI during the 12 months of the review<sup>243</sup>. Furthermore, the rate of recurrent febrile UTI was also not reduced by prophylaxis in a recent Italian study<sup>244</sup>.

#### 1.3.4.2 Reconsidering antibiotics

As discussed above, the applicability of antibiotic therapy for UTI and RUTI is debatable. Indeed, acute, uncomplicated UTI poses minimal risk of progression to tissue

invasion or sepsis; and spontaneous resolution of infection occurs in a large proportion of patients. A Belgium study noted over 30% of patients reporting either symptomatic improvement or cure within two days of starting placebo and this number was over 50% by seven days<sup>223</sup>, suggesting many patients are capable of clearing infection without the aid of antibiotics. Other groups have described clinical cure in the placebo group by day 7 in over 25% of patients, reaching over 50% by the final 4-6 week follow-up<sup>159,213</sup>. Further, placebo-controlled trials of antibiotic treatment for uncomplicated UTI, noted no difference in time to symptom improvement between antibiotic and placebo, with bacteriologic cure rates only marginally better at 4-weeks post treatment<sup>205,245</sup>.

This is not easily explained to patients though who are experiencing the uncomfortable, albeit temporary, symptoms associated with a UTI and physicians often acquiesce to a patient's request for antimicrobial therapy. Such empirical therapy presumes the benefits of rapidly treating those with antibiotic-requiring UTI outweighs the risk of erroneously treating those without UTI or those within whom the infection will self-resolve. Empirical therapy is presumably less costly, as clinicians need not order bacterial culture unless complicating factors are present, and is associated with increased patient satisfaction. However, there are hidden costs associated with this strategy, and the medical field is beginning to question whether the benefits to the individual outweigh individual and societal risks<sup>205</sup>.

At the individual level, antibiotic use is associated with multiple adverse events and has been implicated in damage to the human microbiome. Antibiotics are the most common cause of emergency department visits for adverse drug events in children under 18 and are responsible for up to 20% of all emergency department visits for adverse drug events<sup>246</sup>. Specifically, TMP/SMX is associated with Stevens-Johnson syndrome and toxic epidermal necrolysis requiring hospitalisation<sup>247</sup>. Nitrofurantoin has been linked to the development of erythema multiforme, as well as both acute and chronic pulmonary reactions<sup>247,248</sup>. Further, the fluoroquinolones are known to cause nausea, rash, and abnormal liver function tests<sup>205</sup>. With regards to microbiome effects, most research has focussed on changes to the gut and vaginal microbiomes following antibiotic therapy. Of note, a single antibiotic dose has been shown to decrease the bacterial diversity of the

gut<sup>249</sup>. Antibiotic induced-dysbiosis of these communities is associated with the overgrowth of certain pathogens and the development of disease, such as antibiotic-associated diarrhoea, or vaginal and oral candidiasis. In fact, up to 22% of women that receive antibiotics for uncomplicated UTI will develop vaginal candidiasis<sup>205</sup>.

In addition to these concerns, antibiotics often drop to below minimal inhibitory concentrations (MICs) in the host during treatment, particularly at the beginning and end of therapy, between doses, and throughout antibiotic prophylaxis. At sub-MIC concentrations bacteria adapt the regulation of cellular processes, leading to increases in fitness, virulence, and antibiotic resistance. This phenomena falls under the hormesis “umbrella”, or the concept that the response to a molecule is dependent on the molecule’s concentration. In a pioneering study from the Davies Lab, researchers noted the activity of up to 5% of *Salmonella typhimurium* promoters were modified in response to subinhibitory concentrations of erythromycin and rifampicin<sup>250</sup>. Other groups have taken a less global approach and focussed on specific markers of virulence. Specifically, studies have identified the up-regulation of Panton-Valentine leukocidin by oxacillin and haemolysins by  $\beta$ -lactams in *Staphylococcus aureus*<sup>251,252</sup>, exoproteins by clindamycin in *Streptococcus pyogenes*<sup>253</sup>, as well as type III secretion system protein PrgH and invasion protein by tetracycline in *Salmonella typhimurium*<sup>254</sup>; while downregulation has been shown for Panton-Valentine leukocidin by clindamycin and linezolid in *S. aureus*<sup>251</sup>, streptococcal pyrogenic exotoxin B by clindamycin and linezolid in *S. pyogenes*<sup>253</sup>, and flagellin by mupirocin in *P. aeruginosa* and *P. mirabilis*<sup>255</sup>.

In terms of developing antibiotic resistance, selection by sub-MICs is often associated with low-fitness cost mutations, unlike selection at, or above, the MIC, which is associated with high-fitness costs and comparatively thrive under the selective pressure of high antibiotic concentrations. Subinhibitory concentrations also enrich for bacteria with a high mutation rate, coupled with increases in horizontal gene transfer and recombination; these strains accumulate mutations faster and therefore more rapidly adapt to the environment. Such growth-inhibitory conditions are also associated with an increased frequency of “persister” cells, wherein a subset of the susceptible population

arrests metabolic activity, converting to a slow/non-growing phenotype that is reversible upon removal of the selective pressure<sup>256</sup>.

Given the empirical nature of antibiotic therapy for UTI, there is much concern surrounding the over use and inappropriate prescription of antibiotics to these patients. Notably, published guidelines exist outlining optimal antibiotic selection for UTI, yet the literature continues to demonstrate significant variation in the prescribing practices of physicians<sup>234</sup>. In fact, the Centre for Disease Control (CDC) estimates that up to 50% of all antibiotic prescriptions are unneeded or are not as optimally effective as possible<sup>246</sup>. Furthermore, empirical treatment increases the likelihood that some patients are treated with antibiotics lacking efficacy against the causative uropathogen<sup>234</sup>.

One of the major concerns associated with inappropriate antibiotic use is the increasing prevalence of antibiotic resistance among bacteria, including uropathogens. Notably, many of the bacteria commonly associated with UTI, are on the CDC's list of bacteria identified as antibiotic resistance threats and are either listed as urgent or serious, including *E. coli*, *Enterococcus* sp., *Klebsiella* sp., *Pseudomonas aeruginosa*, and *S. aureus*<sup>246</sup>. Less commonly linked to UTI, but still a known cause and included on the CDC's list are; *Acinetobacter* sp., *Campylobacter* sp., *Salmonella* sp., *Shigella* sp., and *Streptococcus pneumoniae*. Inclusion of so many uropathogens on this list is concerning and suggests that antibiotic therapy for this disease is not being conducted effectively.

Unsurprisingly, a retrospective analysis of over 600 children with first time UTI found no benefit in the use of antimicrobial prophylaxis to reduce the rate of RUTI; however, prophylaxis was associated with increased risk of resistant infections<sup>200</sup>. Furthermore, a prospective study comparing antibiotic prophylaxis with second-generation cephalosporins to TMP/SMX found the second generation cephalosporins increased resistance to both classes of antibiotics, while TMP/SMX increased resistance only to itself<sup>257</sup>. Predictably, TMP/SMX treatment in the previous 3-6 months is an independent risk factor of TMP/SMX resistance in UTI treatment<sup>234</sup>, and resistance to this antibiotic is increasing, in some areas uropathogen resistance is over 20%<sup>209</sup>.

Indeed antibiotic resistance is globally perceived as a looming public health crisis. In the US alone, 2 million people will acquire an infection with bacteria resistant to at least one antibiotic, and 23,000 will die each year as a direct result of these infections<sup>246</sup>. Globally antibiotic-resistant bacteria are estimated to kill 700,000 annually, a number that is predicted to rise to 10 million by 2050<sup>258</sup>. In addition to increased morbidity and mortality, antibiotic resistance can be directly related to increased expenditure. A retrospective Finnish study determined the cost of treating extended-spectrum beta-lactamase (ESBL)-producing bacteria in children with UTI is roughly double the cost of treating a child with an ESBL-negative bacterium<sup>208</sup>.

The antibiotic resistance dilemma is compounded by a number of factors; first and foremost is the development of resistant bacteria, which occurs for each new antibiotic and antibiotic class. Resistance development can be slowed down by antibiotic stewardship; however, bacterial evolution ensures it can never be completely stopped. Secondly, the drug development pipeline is inherently slow, taking up to 8 years to bring a drug from phase I clinical testing to product launch, not including the time spent on drug development<sup>259</sup>. Finally, there is limited investment in antibiotic discovery and development. Estimates vary on the cost of research and development for a new drug, ranging from \$400 million to \$1.5 billion<sup>258,259</sup>. For drugs used in chronic disease, the investment is worth it; however, antibiotics have a limited market, an effective antibiotic will be used for a few days or weeks, and then stopped. Additionally, use of newly developed drugs is often discouraged; they are maintained as drugs of last resort in an effort to curtail resistance development. Thus antibiotic development lacks economic incentives.

A number of programs have been developed in the last few years to address some of these issues including the “New Drugs for Bad Bugs” programme and recent changes to European Medicines Agency policy that reduces clinical trial sizes<sup>260</sup>. However, it will take time for these policies to have affect clinical drug availability. In the interim, alternative treatment strategies should be considered for UTI.



### 1.3.4.3 Ibuprofen as an antibiotic alternative

Multiple antibiotic alternatives for the treatment of UTI have been studied, including functional foods, mucosal vaccines, and bacterial interference strategies. Ours was one of the first groups to suggest bacterial interference may be effective in the prevention of UTIs after recognising a correlation between *Lactobacillus* sp. colonisation of the bladder and decreased risk of UTI<sup>261-263</sup>. Since then, multiple studies have assessed the use of probiotics in the prevention of UTI and have extended to the purposeful colonisation of bladders with ABU strains.

An entirely different approach to UTI treatment is the concept of treating the symptoms, not the infection. This approach is based on the assumption that most UTIs are self-resolving and that symptomology is the impetus for patient antibiotic requests. This approach also builds off the fact that many women do not immediately seek medical attention for UTI, rather they wait for the infection to clear on its own or try to treat with home remedies<sup>264</sup>. Indeed, a number of trials have studied the delay of antibiotic prescription for UTI, these reports comment on delayed symptom resolution and increased time to clearance, yet delay was not associated with serious complications<sup>265</sup>.

Over the last decade, multiple studies have been published comparing the treatment of UTI symptoms to antibiotics<sup>237,264-268</sup>. Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), is often the drug of choice in these studies due to its analgesic, antipyretic, and anti-inflammatory properties. In a pilot study, Bleidorn *et al.*, suggested the use of ibuprofen performed equivocally to ciprofloxacin for treatment of uncomplicated UTI with regards to symptom resolution and course of disease<sup>264</sup>. Secondary antibiotic treatment was required in 33% of the ibuprofen group compared to 18% in the ciprofloxacin, and this difference was not significant. Notably, 66% of patients recovered without the use of antibiotics. Building on this momentum, the same group conducted a double-blind, randomised, multicenter trial of almost 500 patients comparing ibuprofen to fosfomycin<sup>265,266</sup>. Again, 66% of patients receiving ibuprofen recovered without antibiotic treatment; however, non-antibiotic treatment correlated with a higher burden of symptoms. Concurrently, antibiotic prescription for symptom persistence or worsening occurred in 34% of ibuprofen patients compared to 14% of fosfomycin patients, and this

was a significant difference. Interestingly, between days 8 and 15 infection relapse was more common in the ibuprofen group, yet recurrence between days 15 and 28 was more common in the fosfomycin treated group. However, a follow-up of these patients identified no difference in self-reported recurrence between 28 days and six months between the two groups<sup>267</sup>. Further post-hoc analysis of this trial involved the development of a model for use in clinical decision making to determine which patients are likely to succeed using symptomatic therapy<sup>269</sup>, clinical validation of this method remains to be seen.

Conference proceedings of a Swiss trial report similar findings between norfloxacin and diclofenac, wherein patients were more likely to seek reconsultation for UTI symptoms in the diclofenac group (19% vs 9%); however, over 60% of patients receiving diclofenac did not require antibiotics<sup>268</sup>. A further study was undertaken across four sites in Norway, Sweden and Denmark, comparing a higher dose of ibuprofen to mecillinam over the course of three days<sup>237</sup>. Although the study's clinical trials registry lists it as completed, no data is presently available.

Interestingly, the clinical cure rate of ibuprofen patients within each of these studies is generally higher than those reported in placebo studies. This is particularly interesting, as a number of groups have suggested ibuprofen has antimicrobial properties<sup>270,271</sup>. Recently, Obad *et al.* demonstrated ibuprofen mediated growth suppression of Gram-positives including, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Micrococcus luteus*; but not of Gram-negatives (*E. coli*, *P. aeruginosa*, or *Salmonella typhimurium*)<sup>271</sup>. The concentrations identified in these studies are physiologically relevant to human urine, suggesting ibuprofen may have inhibitory effects in the bladder.

## 1.4 Enterococcus

As discussed earlier, enterococci consistently rank within the top three uropathogens in regards to infection incidence, with most enterococcal UTIs being associated with *E. faecalis*. Two species of *Enterococcus*, *E. faecalis* and *E. faecium*, account for the majority of clinical enterococcal infections including medical device-associated

infections, endocarditis, periodontitis, and UTI<sup>272</sup>. First observed as pathogens 100 years ago, *Enterococcus* sp. have since established themselves as a major threat to human health<sup>260</sup>. In addition to their pathogenic potential, enterococci are commensals of the gastrointestinal tract, with *E. faecalis* being the most ubiquitous among species of animals, birds, insects, mammals, and reptiles<sup>273</sup>. In humans, 98% of people are colonised by enterococci<sup>274</sup>. Commensal *E. faecalis* are also considered to offer a health benefit to the host; *E. faecalis* has been used as a commercial probiotic for over 50 years, and commensal *E. faecalis* produce a pheromone capable of killing multi-drug and vancomycin-resistant *E. faecalis* in addition to inhibiting both Gram-positive and negative pathogens<sup>272,275-277</sup>.

A key step in the development of *E. faecalis* colonisation and infection is adherence to biotic or abiotic surfaces accompanied by biofilm development. In fact, most of the virulence factors that have been characterised from *E. faecalis* are biofilm and/or adhesion related. Examples of such factors are the endocarditis and biofilm-associated pili (Ebp), which plays a role in adherence to collagen and is necessary for adherence to fibrinogen<sup>278</sup>; adhesion to collagen of *E. faecalis* (Ace) which binds laminin as well as collagen types I and IV<sup>279</sup>; and aggregation substance (AS) which creates aggregates on the bacterial cell surface that are involved in conjugation and adherence to eukaryotic surfaces<sup>272</sup>. Other virulence factors include the *E. faecalis* endocarditis antigen (EfaA) and extracellular surface protein (Esp) along with the secreted enzymes; gelatinase (GelE), extracellular serine protease (SprE), and cytolysin (CylL). Regulation of virulence is mediated by multiple systems including, the global regulator of virulence (GrvRS, previously EtaRS), vancomycin resistance (vanSR), and fsr-quorum sensing systems (FsrABC).

In addition to biofilm formation and virulence factor production, enterococci are notoriously antibiotic resistant<sup>272,280,281</sup>, a factor of both inherent and acquired features. The CDC included vancomycin-resistant *Enterococcus* (VRE) as a serious domestic antibiotic resistance threat in their 2013 threat assessment<sup>246</sup>, and *Enterococcus faecium* was recently included in the World Health Organization's (WHO) priority pathogens list for research and development of new antibiotics<sup>282</sup>. Across the genus,

*Enterococcus* resistance is relatively low, with approximately 10% of infections demonstrating antibiotic resistance, yet this figure is rising. This is unsurprising given almost 80% of all *E. faecium* isolates from health-care-associated infections are vancomycin-resistant in the US<sup>246</sup>. Enterococcal resistance to last-resort antibiotics, including daptomycin and linezolid, has also started to emerge<sup>260</sup>.

Enterococci are one of the most common causes of sepsis in hospitalised patients<sup>283</sup>. Collectively, the CDC estimates there are 66,000 healthcare-associated *Enterococcus* infections each year in the states; of these 20,000 are vancomycin resistant, and 1,300 deaths are directly attributable to this genus<sup>246</sup>. General properties that have contributed to the emergence of enterococci as nosocomial pathogens include; environmental persistence and associated resistance to clinical disinfection protocols, efficient host colonisation, and an intrinsic ability to withstand a broad-spectrum of antimicrobials. Genomic plasticity has likely also played a role as acquired mobile genetic elements can account for up to 25% of the genome for both *E. faecalis* and *E. faecium*<sup>280</sup>. Of all the species of *Enterococcus*, *E. faecalis* demonstrates the greatest resistance to desiccation, while *E. faecium* is the most tolerant of starvation<sup>273</sup>.

#### 1.4.1 Enterococci in the urinary tract

Enterococcal pathogenesis in the bladder is severely understudied relative to other uropathogens. Most studies of UTI use *E. coli* as a model pathogen, which is understandable given its standing as the most prevalent uropathogen. Using *E. coli* as a guide, enterococcal adherence to and biofilm formation on urothelial cells has been well documented using mouse models of UTI and is thought to be primarily mediated through the Ebp pili, as well as the adhesins, Ace and Esp<sup>21,272,280</sup>. Unsurprisingly, attachment of Gram-negative uropathogens is much better characterised; both *E. coli* and *Klebsiella* sp. have been shown to bind UP proteins and *E. coli* binds  $\alpha_3\beta_1$  integrins to initiate adherence<sup>21</sup>. Enterococcal interactions with UP proteins has not been demonstrated; however, this has been proposed to be mediated through Esp<sup>284</sup>. Further, adherence to macrophages is mediated in part through AS-integrin interactions and enterococcal attachment to the gastrointestinal epithelium involves interactions with GAGs<sup>285,286</sup>, yet these interactions have not been demonstrated within the urinary tract. Similarly, invasion

of the bladder epithelium by *E. faecalis* was only recently reported via *ex vivo* imaging of shed human urothelial cells<sup>287</sup>. This invasive capability was then confirmed *in vitro* using a human bladder epithelial cell line.

Unlike *E. coli*, the most common cause of UTI, *E. faecalis*, generally, does not induce severe symptoms of UTI. On the contrary, many *E. faecalis* UTIs present as low-grade discomfort and urine counts below the classic standard threshold for UTI of 10<sup>5</sup> CFU/mL. However, it should be noted that groups have suggested this standard is too high for *E. faecalis* and that true enterococcal UTI may be caused by a lower bacterial count<sup>288</sup>. An alternative explanation is that most enterococci do not produce potent pro-inflammatory toxins and this is supported by research demonstrating minimal induction of bladder inflammation by *E. faecalis* in a murine cystitis model<sup>280,289</sup>. Unsurprisingly, enterococcal UTI is one of the least likely bacteria to meet standardised diagnostic criteria<sup>214</sup>. Further, *Enterococcus* is prevalent in polymicrobial UTI<sup>290</sup>; given that most polymicrobial isolates are dismissed as contamination, enterococcal UTI may be under-diagnosed.

*E. faecalis* is also a common cause of ABU, with multiple studies identifying it as the second most prevalent organism<sup>199,232</sup>, despite the presence of a number of virulence factors<sup>291,292</sup>. Interestingly, Cai *et al.* found *E. faecalis* in the majority of ABU patients who had a history of UTI but were recurrence free<sup>232</sup>. Of further interest is the finding that treatment of enterococcal ABU increases the incidence of symptomatic UTI in the post-treatment period<sup>291</sup>. Similar results have been found in children receiving antibiotic therapy for UTI; recurrence rates were higher for those with enterococcal UTIs compared to those with Gram-negative UTIs<sup>293</sup>.

In the Wolfe *et al.*<sup>102</sup> study of healthy adult female urine, no enterococci were detected, a trend further demonstrated by Siddiqui *et al.* in their study of healthy female urine<sup>112</sup>. However, a second study by this group positively identified *Enterococcus* in the urine of women with IC/BPS<sup>113</sup>. Further, neurogenic bladder patients tend to demonstrate a urine microbiota that is dominated by *Enterococcus*<sup>114</sup>, suggesting that a demonstrable abundance of these organisms are associated with a diseased, rather than a healthy urinary tract.

## 1.5 Bacterial interactions with the nervous system

### 1.5.1 Sensory properties of the urothelium

Over the last few decades, concepts surrounding urothelium function have vastly changed. Once considered to function purely as a barrier against urine, the understanding of urothelial functions has evolved to include metabolic activity and sensation of both chemical and mechanical stimuli<sup>6,87</sup>. Sensory input is communicated to nerves, interstitial, smooth muscle, vascular, and inflammatory cells through urothelial release of effector molecules<sup>38</sup>. In turn, the urothelium is responsive to signals from these systems; de-innervation of the bladder, for example, results in acute structural disruption of the urothelium and subsequent loss of barrier function<sup>31</sup>. This “sensory web” regulates urothelial permeability, contraction and relaxation of the detrusor, and host sensation of bladder fullness<sup>16</sup>.

The neuron-like properties of the urothelium are accorded through a variety of receptors including those classically associated with the nervous or muscular systems, such as purinergic, adrenergic, and cholinergic receptors. In some cases, the density of these receptors is quite high, such as in pigs, where the mAChR population is 1.5 times greater in the urothelium than the detrusor<sup>294</sup>. Additionally, protease-activated ion channels, epithelium sodium channels, and members of the TRP family are found within this tissue layer<sup>8,16</sup>. Bladder epithelial cells are also able to respond to hormones, neuropeptides, and chemokines among other molecular signals<sup>38</sup>. Effector molecules released at either the apical or basolateral surface mediate the urothelial response.

In a healthy bladder, bladder-filling increases pressure on the urothelium, which induces urothelial release of neuroactive effectors such as ATP, NO, ACh, adenosine, and prostaglandins (PGs)<sup>6,79</sup>. Although the exact mechanism has not yet been characterised, urothelial ATP is thought to activate P2X<sub>3</sub> receptors on suburothelial sensory afferents<sup>8,77</sup>, communicating bladder fullness to the brain. The presence of P2X receptors on the serosal surface of urothelial cells suggests the ATP signal is propagated through the urothelial layer, which may play a dual role; relay of the signal to afferent nerves and

expansion of UC apical membrane surface area through stimulation of P2X receptor-mediated vesicular traffic<sup>16</sup>. Further, the stimulus may be amplified through autocrine signalling or ICs acting as intermediaries between the urothelium and nerves or SMCs. Other response mechanisms include the release of NO following activation of urothelial TRPV1,  $\alpha$ -AR or  $\beta$ -AR and release of an as-of-yet-unidentified inhibitory factor in response to autocrine or paracrine ACh<sup>15,76,79,87,295</sup>.

Further support for the sensory functions of the urothelium can be found in pathological states, wherein alterations to urothelial structure or receptor expression patterns correlate with bladder pathology. Dysregulation of urothelial mAChRs has been linked to detrusor overactivity and increased release of ACh during bladder filling associated with OAB<sup>2,87,88</sup>. Alterations in urothelial expression of TRP family and purinergic receptors has been observed in IC/BPS, OAB, bladder outlet obstruction (BOO), and detrusor overactivity. Similarly, increased ATP release has been noted in response to stretch and electrical field stimulation in urothelial cells isolated from patients with functional bladder disorders. Correspondingly, urinary ATP is increased in patients with IC, OAB and other functional bladder disorders<sup>38</sup>.

### 1.5.2 Potential for bacterial interactions

Many bacteria have been documented to interact with the nervous system<sup>154,296-299</sup>. In most such cases this leads to the development of disease, for example, those caused by neurotoxins, such as botulinum (botulism) or the invasion of neural cells as is the case for *Mycobacterium leprae*. To date, neuronal interactions with bacteria have focused upon aggressive nervous system pathogens, though now we are beginning to understand that bacteria throughout the body may have other distinct but subtle effects on the nervous system.

Bacteria have been documented to produce and interact with classical neurotransmitters for over 100 years, yet direct applications to host homeostasis were rarely considered, with the exception of infection, until relatively recently. Indeed, bacteria are known to produce  $\gamma$ -amino butyric acid (GABA), NE, ACh, tyramine, tryptamine, serotonin, and many other neuroactive substances<sup>300-304</sup>. Unfortunately, most studies assessing bacterial

production of these molecules are completed by food microbiologists and are thus primarily studied under food storage or production conditions. Strategies to reduce histamine associated scombroid poisoning or tyramine induced hypertensive crises in persons on monoamine oxidase inhibitors abound within the literature<sup>305-308</sup>. However, the potential for bacteria to produce these molecules in the human host and how this might alter the host response or progression of disease has been given minimal consideration.

To date, most research has focussed on the microbiota-gut-brain axis, the theory that gut bacteria interact with the enteric nervous system (ENS), which in turn communicates with the brain to modulate host homeostasis or behaviour<sup>154,297,298</sup>. Given the gut is highly innervated and one of its primary functions is nutrient absorption, it stands to reason that metabolites produced by the gut microbiome would signal through the ENS. Notably, species of *Lactobacillus* and *Enterococcus* are capable of GABA production, and oral administration of *Lactobacillus rhamnosus* JB-1 reduces anxiety- and depression-related behaviour in mice through GABAergic signalling<sup>309-311</sup>. Abnormal behaviour has also been noted in germ-free mice<sup>296,312</sup>, which is likely due to a loss of microbial metabolites.

In terms of the bladder, intravesical administration of chemical stimuli induces changes in bladder capacity and activity. Thus bacterial production of neuroactive substances in the bladder is likely to affect independent of damage to the bladder's barrier. Intravesical instillation of vanilloids reduces symptoms of detrusor over activity and IC, PGE<sub>2</sub> or ATP induces bladder hyperactivity, capsaicin reduces the desire to void and increased bladder capacity in patients with hypersensitivity, and nerve growth factor (NGF) sensitises the afferent nerves to subsequent mechanical stimuli<sup>6,16</sup>. While the exact mechanisms of action are unknown, urothelial sensory properties and innervation of the urothelium have been proposed. Groups have also demonstrated cytoplasmic projections that anchor the UCs to the basement membrane, which could provide a direct link to the suburothelial plexus or ICs. There is also reason to believe bacteria may indirectly produce neuroactive substances through natural response mechanisms, for example, the host response to bacteria involves inflammation and PGs are mediators of inflammation. However, PGs have also been shown to induce bladder contraction<sup>2</sup>. Interestingly, PGE<sub>2</sub> is higher in the



urine of patients with acute UTI compared to healthy controls, and this correlates with symptom onset and duration<sup>313</sup>.

Bacterial communication with the host through metabolite intermediates is certainly not something unique to the bladder or gut. Researchers at Johns Hopkins suggest microbially-derived short-chain fatty acids act as ligands for G-protein-coupled receptor 41 and olfactory receptor 78 to modulate blood pressure control at the level of the kidney<sup>314</sup>. Bacteria have co-evolved with their mammalian hosts, and are constantly exposed to eukaryotic communication. Thus, the hypothesis that bacteria also use these systems as a means of communication intra-and inter-kingdom is not a leap. Indeed, GABA is a chemotactic agent for pseudomonads, increases the bacterial cytotoxicity of *P. aeruginosa* and induces lipopolysaccharide (LPS) rearrangement in *P. fluorescens*<sup>300,315</sup>.

The catecholamines are also found throughout nature and elicit responses in many species of bacteria. Norepinephrine enhances the growth of *P. aeruginosa* in iron-limited minimal media by acting as a siderophore and represses production of virulence factors negatively regulated by iron<sup>316</sup>. The growth of *E. coli* O157:H7 and *S. enterica* is stimulated by NE, dopamine (DA), and epinephrine (EPI); while *Yersinia enterocolitica* only responded to NE and DA<sup>317</sup>. Similarly, NE increased the growth, motility, and host cell invasion of *Campylobacter jejuni* under iron-limited conditions<sup>318</sup>. Yet, exposure of *Salmonella enterica* serovar typhimurium to EPI reduced the expression of an antimicrobial peptide resistance operon and concomitantly increased polymyxin B susceptibility<sup>319</sup>. Further, EHEC exposure to EPI and NE is associated with increased motility, biofilm formation, and attachment to porcine colonic mucosa and bovine ileum<sup>320-322</sup>.

Alternatively, bacterial infection is known to alter the urothelium<sup>28</sup>, which has the potential to expose underlying, immature cell layers and/or tissues to direct interaction with bacterial metabolites. Such disorganisation may also extend to the ATP-mediated vesicular transport system, predisposing the host to urgency and frequency.

### 1.5.3 Linking bacteria to bladder dysfunction

Symptoms associated with loss of micturition control or dysfunctional bladder sensations are associated with many urinary disorders, urgency and frequency are experienced by patients with OAB, and idiopathic pain is a hallmark of IC/BPS. However, these symptoms are also present during UTI. While inflammation is expected to play a role in these pathologies, our group proposes bacteria are capable of subterfuge – use of the host response to bacterial advantage. A good model of this is the difference between ABU and UTI. Patients with ABU meet the diagnostic cut-off for UTI, which is presumably caused by an equally virulent strain, yet experience no symptoms of UTI. Thus, the potential exists for the bacteria to either elicit a weaker immune response in this state or interfere with the regular signalling systems within the bladder, “dulling” the host sensation.

Of note, patients with RUTI, OAB, IC/BPS, SCI, and BOO all experience LUTS and exhibit similar pathologies including an increase in urothelial apoptosis and decrease in urothelial E-cadherin expression, indicators of impaired urothelial differentiation<sup>30</sup>. Further to this point, UPII KO mice demonstrate voiding patterns typical of IDO, which could be a factor of increased urothelial permeability and/or dysfunctional smooth muscle tissue<sup>27,323</sup>. Observations have also been made in regards to reduced sensitivity of UPII and UPIIIa KO mice to carbachol-induced contractions of denuded bladder strips<sup>323</sup>. Given that the urothelial layer has been removed from these strips prior to contractile studies, it is unlikely that the UP proteins are actively contributing to the change in sensitivity. Thus, reduced sensitivity may be a result of mAChR and nicotinic acetylcholine receptor (nAChR) “overexposure” to urinary metabolites due to increased urothelial permeability, which also occurs during UTI.

In contrast, the contractile response to carbachol was significantly higher in denuded detrusor strips from paediatric patients with a history of RUTI relative to age-matched controls, which was suggestive of infection-induced upregulation of cholinergic receptors or neurons<sup>324</sup>. Importantly, IDO has been correlated with increased CGRP and SP immunoreactivity in the bladder and OAB linked to increased urinary NGF and brain-derived neurotrophic factor (BDNF)<sup>67,325</sup>. Inflammation is also associated with increased

expression of neuropeptides in the bladder and urine<sup>38,326</sup>. However, as most studies focus on acute inflammation, the length of time this persists for remains unknown, yet the links between UTI and subsequent bladder dysfunction seem to suggest that dysfunction is long-term in some patients.

In particular, multiple studies have documented UTI episodes prior to the onset of IC/BPS and oversensitivity has been proposed as a mechanism for multiple chronic pain syndromes<sup>327</sup>. Similar trends have been found in the gastrointestinal tract; the density of SP and CGRP nerves was higher in *H. pylori* infected mice, remaining higher after *H. pylori* eradication<sup>328</sup>. These changes were coupled with a decrease in ACh release upon electrical field stimulation, suggesting *H. pylori* induced both morphological and functional changes to the nervous system. Furthermore, between 10 and 30% of patients with acute gastroenteritis will develop symptoms of irritable bowel syndrome<sup>329</sup>. The applicability of this to the bladder is supported by rat studies that demonstrate the relationship between early life inflammation and the persistence of SP upregulation into adulthood, which is associated with decreased bladder volume and a concomitant increase in micturition frequency<sup>330</sup>. In humans, a retrospective study comparing women with RUTI to those with stress urinary incontinence and no history of RUTI found that women with a history of bladder infection had a greater urinary frequency, lower average void volumes and a lower threshold of bladder sensitivity<sup>327</sup>. Thus, it is likely that bacteria either directly, through production of neuroactive substances, or indirectly, through host response to infection, modulate the sensitivity of the bladder to stimuli.

## 1.6 Scope and purpose

At the commencement of my doctoral studies, enterococcal invasion of the urothelium had not yet been demonstrated, the urinary microbiota not yet described, and microbial endocrinology was a fledgeling field of research. *E. faecalis* remains a major cause of UTI, and the rates of antibiotic resistance are increasing at a steady rate. Thus the purpose of this thesis was to increase understanding of enterococcal UTI, in particular, the response of *Enterococcus* to antibiotic prophylaxis *in vitro* and *in vivo* and enterococcal communication with the bladder.

The second chapter addresses the response of *E. faecalis* to nitrofurantoin and TMP/SMX, two of the most commonly prescribed antibiotics. Unexpectedly, nitrofurantoin induced a major increase in enterococcal attachment to urothelial cells. We further explored the transcriptional response of *E. faecalis* to nitrofurantoin. We also investigated IBU as a candidate antibiotic potentiator.

The third chapter incorporates a clinical study we undertook in London, Ontario to study the effects of TMP/SMX and nitrofurantoin on bacteria *in vivo*. Our study was to be the first description of the urinary microbiota in the paediatric population, the first longitudinal assessment and the first to study changes in the urinary microbiota as a response to antibiotic prophylaxis. Our study demonstrated the presence of uropathogens in urine throughout the study period, regardless of antibiotic status and absence of UTI symptomology.

In the fourth chapter, we discuss the neuromodulatory properties of *E. faecalis*. Our initial proposal involved the characterisation of GABA production by enterococci. However, as we moved forward with the experiments, we switched from a colourimetric assay to a mass spectrometry method. Through the use of targeted mass spectrometry, we were able to identify enterococcal production of neuroactive molecules *in vitro* under conditions mimicking the bladder environment.

At the culmination of my thesis, *Enterococcus* remains a relatively understudied pathogen in the context of UTIs; however, this has started to change. Throughout this thesis, I have questioned the efficacy of antibiotic prophylaxis for RUTI, particularly in patients with a history of enterococcal UTI. Further, I demonstrate the potential for *E. faecalis* to interact with the sensory system of the bladder. It is my sincere hope that others use this thesis as a starting point for further hypothesis-driven investigations.

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## Chapter 2

### 2 Nitrofurantoin increases bacterial attachment to urothelial cells

#### 2.1 Introduction

Urinary tract infections (UTIs) can be associated with decreased quality of life, psychological distress, and loss of functional hours<sup>1</sup>. Females are significantly more likely than males to experience UTI, which has been attributed to the difference in urethral length<sup>2</sup>. Notably, women experience an average of 6.1 symptomatic days, 2.4 days of decreased activity and 0.4 days of bed rest per symptomatic episode<sup>3</sup>, which is a huge economic loss when considering the high incidence of this disease. Further, recurrence rates are quite high; up to 25% of women with acute UTI will recur within 12-months, and 22% will go on to develop recurrent UTI (RUTI)<sup>4</sup>, which is defined as three or more symptomatic UTIs in a 12-month period or two or more within six-months. More conservative estimates suggest up to 5% of women experiencing their first UTI will develop RUTI<sup>1</sup>. In children, recurrence rates in primary care are approximately 12% per year for both males and females<sup>5</sup>, while referral and emergency department data indicates 20-48% will recur within 12 months<sup>6,7</sup>.

At present, antibiotic therapy is the standard of care for both UTI and RUTI<sup>8-10</sup>; however, such widespread use has become contentious. Antibiotic resistance has been linked to infection recurrence in 5-10% of women, and this figure rises to 50% following antibiotic prophylaxis<sup>11</sup>. In children, antibiotic prophylaxis is often used in RUTI as a strategy to reduce the risk of pyelonephritis, renal scarring and febrile UTI<sup>6</sup>; yet clinical studies do not demonstrate these benefits<sup>7,12,13</sup>. Rather, there is either a slight benefit in prevention of symptomatic UTI or no reduction<sup>6,14</sup>.

Furthermore, sub-minimal inhibitory concentrations (MICs) of antibiotics are associated with bacterial adaptation of cellular processes, increasing bacterial fitness, virulence, and antibiotic resistance. Our lab has previously documented increased adherence to urothelial cells and bladder invasion by *Staphylococcus saprophyticus* and *Escherichia*

*coli* following exposure to sub-MIC ciprofloxacin<sup>15,16</sup>. Similar effects have been noted for a variety of antibiotic classes and bacteria<sup>17-20</sup>. Sub-inhibitory concentrations are often encountered throughout antibiotic prophylaxis, yet research into the effects of nitrofurantoin and trimethoprim-sulfamethoxazole (TMP/SMX), two of the most commonly prescribed prophylactic agents, is limited.

Concerns with antibiotic usage, combined with the high rate of spontaneous cure associated with UTI, have paved the way for studies into alternative UTI therapeutics. The clinical intent for UTI is primarily cessation of symptoms; antibiotics quickly achieve this. However, ibuprofen (IBU) presents a viable alternative for symptom management, providing the relief patients desire, while the immune system clears the infection. As one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs), IBU has a well-characterised safety profile and is known for its analgesic, antipyretic, and anti-inflammatory properties that are mediated through nonspecific inhibition of cyclooxygenases. In clinical studies, IBU compared equivocally to ciprofloxacin for the management of uncomplicated UTI<sup>21</sup>, yet did not perform as well when compared to fosfomycin<sup>22-24</sup>. In a murine model of acute UTI, NSAID administration reduced bacterial burden and prevented mucosal wounding<sup>25,26</sup>. The general belief is that IBU “masks” the symptomology, allowing the host’s immune system to clear the infection independent of antibiotics; however, clinical cure rates, defined as loss of symptoms, are generally higher in the IBU arm of UTI studies relative to the placebo arm of other studies<sup>22-24,27</sup>, indicating it may have antimicrobial properties. Indeed, multiple reports exist of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, and *Micrococcus luteus* growth suppression by IBU *in vitro*<sup>28,29</sup>. Ibuprofen is metabolised by cytochrome P450 (CYP) enzymes to two major metabolites, 2-carboxyibuprofen (CIBU) and 2-hydroxyibuprofen, and two minor metabolites 3-hydroxyibuprofen and 1-hydroxyibuprofen, which are ultimately excreted in the urine<sup>30,31</sup>.

Given the high rate of antibiotic resistance among uropathogens and its relation to sub-MIC antibiotics, the study of bacterial adaptation to antibiotics at these concentrations is a priority. *Enterococcus faecalis*, one of the most common uropathogens<sup>32-34</sup>, is well

known for innate and acquired antibiotic resistance properties, yet the bacterium's response to sub-MIC antibiotics is understudied. Further, the effects of IBU on *E. faecalis* virulence are unknown.

In this chapter, we assessed the role of nitrofurantoin, TMP/SMX, IBU and CIBU on enterococcus growth and adherence characteristics, as well as the potential for synergy between the antibiotics and IBU. We hypothesised that sub-MICs of nitrofurantoin and TMP/SMX would increase the attachment of *E. faecalis* to urothelial cells. We further hypothesised that addition of IBU or CIBU would reduce these effects, potentiating the antibacterial properties of nitrofurantoin and TMP/SMX.

## 2.2 Materials and methods

### 2.2.1 Bacterial strains, eukaryotic cell lines, media, and chemicals

Bacterial strains used throughout these experiments are listed in Table 2-1. Strains were routinely cultured without aeration at 37°C, in brain-heart infusion broth (BHI; Difco Laboratories Inc., Franklin Lakes, NJ, USA) for *E. faecalis* and Luria-Bertani (LB) broth for *E. coli*. Growth experiments were conducted with Mclean's Artificial Urine (AU)<sup>35</sup>. Minimal inhibitory concentration studies were completed using cation-adjusted Mueller Hinton broth (CAMHB; Difco) for both *E. faecalis* and *E. coli*.

A human urinary bladder epithelial cell line, 5637's (ATCC HTB-9), initially isolated from a 68-year-old male with grade II carcinoma, was acquired from the American Type Culture Collection (ATCC). Urothelial cells were maintained in T75 flasks, in a 5% CO<sub>2</sub> tissue culture incubator at 37°C in Roswell Park Memorial Institute medium 1640 (RPMI; Gibco, Burlington, ON, CAN) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM glutamine (Gibco) and penicillin-streptomycin (Gibco) at 100 U/mL and 100 µg/mL respectively. Supplemented-RPMI without the addition of antibiotics will herein be referred to as infection media.



**Table 2-1. Strains used in this study.**

Strain	Initial Isolation	Source
<i>E. faecalis</i>		
A256	Urine of patient with RUTI and urosepsis <sup>36</sup>	Our laboratory
1131	Uropathogen <sup>37</sup>	Our laboratory
1396	Female with UTI <sup>38</sup>	Our laboratory
23241	Urine of patient with pyelonephritis <sup>39</sup>	ATCC <sup>a</sup>
29212 <sup>b</sup>	Urine <sup>40</sup>	ATCC <sup>a</sup>
OG1X	Derivative of oral isolate 2SaR <sup>41</sup>	Our laboratory
5024-8	Gastrointestinal	Our laboratory
19433 <sup>b,c</sup>	Documentation not available	ATCC <sup>a</sup>
33186 <sup>b</sup>	Documentation not available	ATCC <sup>a</sup>
<i>E. coli</i>		
UTI89	Patient with UTI <sup>42</sup>	Hultgren laboratory
CFT073	Urine of patient with pyelonephritis <sup>43</sup>	ATCC <sup>a</sup>

<sup>a</sup>American Type Culture Collection.

<sup>b</sup>Strain often used in quality control testing<sup>44</sup>.

<sup>c</sup>Type strain<sup>45</sup>.

Cells were routinely passed when confluency reached greater than 80%. To pass cells, the monolayer was gently washed with warm phosphate buffered saline (PBS) followed by trypsinization for 10 minutes at 37°C and 5% CO<sub>2</sub> by addition of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco). Cells were dislodged with gentle tapping and trypsin inactivated by the addition of cell culture media. For cell maintenance, cell suspensions were reconstituted 10-fold in a new T75 flask with fresh supplemented-RPMI. For experiments, an aliquot of the cell suspension was mixed 1:1 (v/v) with 0.4% trypan blue (Invitrogen, Carlsbad, CA, USA) and 10 µL of sample mixture added to the chamber port of a Countess Cell Counting Chamber Slide (Invitrogen) for use with a Countess Automated Cell Counter (Invitrogen). Only viable cell counts were considered. Cell suspensions then underwent centrifugation at 3,000 rpm for 6 minutes, were reconstituted in infection media, and subsequently seeded to 24-well tissue-culture treated plates (Sarstedt, Nümbrecht, Germany) at  $1 \times 10^5$  cells/mL. Plates were incubated as above until the monolayer reached confluency, at approximately 48 hours.

Solutions of both sulfamethoxazole and trimethoprim were prepared separately and combined prior to use at a ratio of 1:19 (TMP/SMX)<sup>46</sup>. Sulfamethoxazole (Sigma-Aldrich, St. Louis, MO, USA) was solubilised in half the final volume of ddH<sub>2</sub>O with the addition of 2.5M NaOH until dissolved; additional ddH<sub>2</sub>O was added to a final concentration of 50 mg/mL. Similarly, trimethoprim (Sigma-Aldrich) was dissolved in 0.05M HCl and water added to a final concentration of 20 mg/mL. Nitrofurantoin (Sigma-Aldrich) was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich) at a stock concentration of 20 mg/mL. Gentamicin (Sigma-Aldrich) stock solutions were prepared in water to 50 mg/mL. Ibuprofen (Galenova, St-Hyacinthe, QC, CAN) was prepared at a stock concentration of 0.1M (20.6 mg/mL) in ethanol (Sigma-Aldrich) for bacterial growth and biofilm formation assays; and 100 mg/mL in methanol (Sigma-Aldrich) for co-culture experiments. Carboxyibuprofen (Toronto Research Chemicals, Toronto, ON, CAN) was prepared at a stock concentration of 2.5 mg/mL in DMSO (Sigma-Aldrich). All stock solutions were filter sterilised prior to storage at -20°C.

### 2.2.2 Minimal inhibitory concentration determination

Minimal inhibitory concentrations (MIC) for TMP/SMX, nitrofurantoin, and IBU were determined according to CLSI standards<sup>44</sup>. In brief, isolated colonies were suspended in CAMHB and incubated overnight at 37°C without aeration. Stationary phase cultures were then diluted 1/100 in fresh CAMHB for incubation at 37°C until culture turbidity was equivalent to a 0.5 McFarland standard<sup>47</sup>. Turbid cultures were diluted 1/20 in fresh CAMHB and 10 µL of suspension added to pre-made antibiotic dilution plates. Based on the 0.5 McFarland standard, the final bacterial concentration was approximately  $5 \times 10^5$  CFU/mL. Inoculated plates were incubated at 37°C for 18-24 hours, at which point growth was assessed using an Eon microplate spectrophotometer (BioTek, Winooski, VT, USA). The MIC was determined as the lowest concentration to completely inhibit bacterial growth. The cutoff is less strict for TMP/SMX, as growth media may contain antagonists, thus the MIC was defined as the lowest concentration to reduce growth by greater than 80% compared to the growth control.

Antibiotic dilution plates were prepared in advance and stored at -20°C until the day of the experiment. Plates were prepared as a series of  $2 \times$  antibiotic dilutions in CAMHB to a final volume of 100 µL/well. Antibiotic vehicle controls were included in the plates, as were both positive and negative growth controls. Antibiotics were regularly assessed against the quality control strains, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922, to ensure consistent results.

### 2.2.3 Growth curves

Growth inhibition by IBU and CIBU was assayed in 96-well plates that were prepared as serial dilutions of the compound of interest with stationary-phase bacteria added at a final dilution of 1/100. Plates were incubated for 24 hours at 37°C with optical density (OD) readings at 600 nm every 15 minutes using an Eon microplate spectrophotometer (BioTek). A range of both IBU and CIBU were assayed, between 200 and 0.02 µg/mL and 25 and 0.025 µg/mL respectively. These concentrations were selected based on solubility in the respective media and urinary availability reported in the literature<sup>30,48-50</sup>.

## 2.2.4 Bacterial adhesion and invasion of urothelial cells

### 2.2.4.1 Co-culture of bacterial and urothelial cells

Bacteria were prepared for co-infections as follows; overnight broth cultures (stationary phase) were reconstituted 100-fold to supplemented infection media (pre-treatment) and incubated to mid-exponential phase (4-hours) at 37°C and 5% CO<sub>2</sub>. Exponential phase cultures were then diluted to a final concentration equivalent to a multiplicity of infection (MOI) of 25:1 bacteria per host cell in appropriately supplemented infection media (co-culture). For IBU and CIBU only experiments, no supplements were added to pre-treatment media, and IBU and CIBU were added directly to the co-culture media. For experiments assessing bacterial attachment in the presence of antibiotic and IBU or CIBU, pre-treatment media was supplemented with the appropriate vehicle or antibiotic at the MIC,  $\frac{1}{2}$  MIC,  $\frac{1}{4}$  MIC, or  $\frac{1}{8}$  MIC. Following pre-treatment, bacterial cells were diluted in fresh infection media supplemented with the appropriate antibiotic or vehicle and either IBU, CIBU, or vehicle. Thus, bacteria were exposed to IBU and CIBU only when in the presence of urothelial cells, while exposure to the antibiotic occurred prior to and throughout urothelial co-culture. Ibuprofen was added to a final concentration of 1000, 200, 100, or 10 µg/mL, while CIBU was added to a final concentration of 10 or 1 µg/mL. Ibuprofen and CIBU concentrations are based on urinary values reported within the literature<sup>30,48-50</sup>. Higher concentrations of IBU caused the media to precipitate and the cost of CIBU was prohibitive towards replicates at higher concentrations. Pre-treatment only experiments were completed with exponential phase cultures diluted in infection media without antibiotics. The MOI was calculated based on the urothelial cell count from a representative well. Cell counts were determined as described above.

Confluent monolayers were washed three times with warm PBS, followed by the addition of 500 µL of the prepared bacterial suspension. Co-cultures were incubated at 37°C and 5% CO<sub>2</sub> for 4 hours.

### 2.2.4.2 Determination of adhesion and invasion

Internalisation counts were determined as follows; following a 4-hour incubation, the co-cultures were washed three times with 500 µL warm PBS followed by 500 µL

infection media supplemented with 200 µg/mL gentamicin, which we confirmed was lethal to non-internalized bacteria. The co-culture was incubated for an additional two-hours to kill any non-internalised bacteria. Following the kill-incubation, the co-culture was washed three times with 500 µL warm PBS. The urothelial cells were then lysed by the addition of 500 µL 0.1% Triton X-100 (Sigma-Aldrich) in PBS at 37°C for 15 minutes. Bacterial counts were acquired by serially diluting the cell lysate ten-fold in PBS, spot-plating 10 µL to agar plates, and counting the resulting colonies.

For adherent cell counts, wells were washed three times with PBS after the 4-hour co-incubation, then immediately lysed and plated as described for internalised cell counts. Total cell counts were acquired after the 4-hour co-incubation by the direct addition of 5 µL 10% Triton X-100 in PBS, the remainder of the cell lysis and plating was as described. Thus for each condition three wells were required per replicate, one each of internalisation, adherence, and total bacterial counts; for experiments assessing attachment only, an internalisation well described above was not included.

Internalised and adherent cell populations are represented as a percentage of the total cell population; the internalised population was considered negligible in the calculation of adherent populations.

## 2.2.5 Distribution of target genes in *E. faecalis*

### 2.2.5.1 Isolation of DNA

Genomic DNA was isolated from strains of *E. faecalis* using InstaGene Matrix (Bio-Rad Laboratories, Mississauga, ON, CAN) according to the manufacturer's instructions. Resultant DNA was stored in the matrix at -20°C and centrifuged at 10,000 rpm for two minutes prior to use.

### 2.2.5.2 Primer design and PCR conditions

Target genes were selected to characterise the genetic potential of *E. faecalis* isolates available within our collection and gene expression study based on major virulence factors and putative nitroreductases genes, as well as other genes that have been documented to be differentially regulated under antibiotic stress or by growth in pooled

human urine relative to laboratory growth medium<sup>51-54</sup>. Primers were designed using Primer-Blast<sup>55</sup>, with primer-dimer detection by ThermoFisher Scientific's Multiple Primer analyzer (Thermo Fisher Scientific, Waltham, MA, USA) and Beacon Designer (Premier Biosoft, Palo Alto, CA, USA) to assess for secondary structures. Prospective primers were only accepted with  $\Delta G$  less than -6 and were synthesized by Invitrogen Life Technologies (Table 2-2).

PCR reactions were performed in a total volume of 25  $\mu\text{L}$ ; 1  $\times$  PCR buffer (Invitrogen), 20  $\mu\text{g}/\mu\text{L}$  bovine serum albumin (Sigma-Aldrich), 10  $\mu\text{M}$  deoxyribonucleoside triphosphates (Invitrogen), 10  $\mu\text{M}$  forward and reverse primer, 50 nM  $\text{MgCl}_2$  (Invitrogen), 1 U Taq-Polymerase (Invitrogen), 1  $\mu\text{L}$  genomic template. PCR conditions were as follows; 94°C for 3 minutes, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C, 60 seconds at 72°C, and a final elongation of 5 minutes at 72°C. Products were visualised by ethidium bromide staining of 2% agarose gels.

## 2.2.6 Bacterial gene expression

### 2.2.6.1 RNA Extraction

Following either a 1-hour or 4-hour incubation, 1 mL RNAProtect Bacteria Reagent (Qiagen, Toronto, ON, CA) was added to each well and incubated at room temperature for 10 minutes. The contents of the well were then transferred to a microcentrifuge tube and centrifuged at room temperature for 5 minutes at 5000  $\times$  g. The supernatant was discarded, and the pellet resuspended in 500  $\mu\text{L}$  lysis buffer followed by incubation at 37°C for 30 minutes and subsequent centrifugation at 4°C and 5000  $\times$  g for 5 minutes. The supernatant was discarded, and 500  $\mu\text{L}$  TRIzol reagent (Invitrogen) added to the microcentrifuge tube. Cell lysate was stored at -20°C until processed using the PureLink RNA MiniKit (Invitrogen) as per the manufacturer's instructions. Lysis buffer was prepared fresh on the day of the experiment as follows, 20 mg/mL lysozyme (Sigma-Aldrich) and 25 U/mL mutanolysin (Sigma-Aldrich) in Tris-EDTA buffer at pH 8.0.

In brief, 200  $\mu\text{L}$  chloroform (Sigma-Aldrich) was added to the microcentrifuge tube and shaken vigorously, followed by an additional 500  $\mu\text{L}$  TRIzol reagent. The solution was incubated at room temperature for 3 minutes followed by centrifugation at 4°C and

12,000 × g for 15 minutes. The upper phase was transferred to a fresh microcentrifuge tube, followed by the addition of 100% ethanol (Sigma-Aldrich) at a 1:1 ratio. This mixture was then vortexed and transferred to a Spin Cartridge, which was centrifuged at room temperature and 12,000 × g for 15 seconds. The column was washed with Wash Buffer I, followed by PureLink On-Column DNase treatment (Invitrogen) for 15 minutes at room temperature. The product was washed twice with Wash Buffer II, and eluted in 30 µL RNase-free water. The resulting RNA was converted to complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Unused RNA was flash frozen and stored at -80°C.

#### 2.2.6.2 Quantitative PCR

Quantitative PCR experiments were completed using *Power* SYBR Green PCR Master Mix (Applied Biosystems) on the QuantStudio 5 Real-Time PCR System (Applied Biosystems). Primer efficiency was determined against genomic DNA from *E. faecalis* 1131; DNA was diluted five-fold and qPCR completed in triplicate. Primers that did not amplify, or those demonstrating an efficiency above 110% or below 90% were not used further (Table 2-2). Initial data analysis and quality control assessment was completed using the QuantStudio Design and Analysis Software v1.4.1 (Applied Biosystems). Data is presented as relative quantification according to the  $\Delta\Delta C_T$  method, wherein vehicle was used as the comparator with both *23S rRNA* and *recA* as endogenous controls.

#### 2.2.7 Statistical analysis

All experiments were completed in biological and technical triplicates. Results in the figures are presented as mean per group ± standard deviation. To test for significance in the final optical density of the growth curves and attachment/invasion we analysed the data using one-way analysis of variance (ANOVA) followed by post hoc Dunnett's multiple comparisons tests. Bacterial relative gene expression analyses were completed using two-way ANOVA followed by post hoc Dunnett's multiple comparisons tests. Pearson's correlation coefficients were used to test for correlation within the data. Data analysis was completed using GraphPad Prism v7.0a for Mac OS X (GraphPad

Software, San Diego, CA, USA). Results were considered significant as follows; \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .



Table 2-2. Primers used in this study.

REFERENCE	Name	Gene target	Symbol*	Locus tag	Primer Sequence (5'→ 3')		Efficiency
					Forward <sup>†</sup>	Reverse	
23S ribosomal RNA			23S	EF2048	CCTATCGGCCTCGGCTTAG <sup>54</sup>	AGCGAAAGACAGGTGAGAAT CC	100.71
Recombinase A			<i>recA</i>	EF3171	GCAACGAAATGGTGGAACAG <sup>56</sup>	AAGGCATCGGCAATCTCTAA G	103.00
<b>ADHESION AND COLONISATION</b>							
Adhesin to collagen of <i>E. faecalis</i>			<i>ace</i>	ND <sup>*</sup>	GGAGAGTCAAAATCAAGTACGTTGG TT <sup>57</sup>	TGTTGACCACCTTCCTTGTGCGA T	103.77
Aggregation substance			<i>asaI</i>	EFA0047	GATACAAAAGCCAAATGTCGTTTCCT <sup>54</sup>	TAAAGAGTCGCCACGTTTCAC A	No Amp <sup>§</sup>
Endocarditis-and-biofilm-associated pilus, subunit A			<i>ebpA</i>	EF1091	CAACAACACACCAGGGCTTTT <sup>58</sup>	ACCGGACCAGTCAACGACTA AG	106.40
Endocarditis-and-biofilm-associated pilus, subunit B			<i>ebpB</i>	EF1092	CGTACAGGCGGCAAGTCTTT <sup>58</sup>	AGGTATTCCCCCGCTTGATTT	106.74
Endocarditis-and-biofilm-associated pilus, subunit C			<i>ebpC</i>	EF1093	GCGGCACACTAAAAATTCGTTTA <sup>58</sup>	GTCGTCGGTATGACCCGTTATC A	104.55
Endocarditis specific antigen			<i>efaA</i>	EF2076	TGGGACAGACCCCTCACGAATA <sup>54</sup>	CGCCTGTTTCTAAGTTCAAGC C	109.52
PavA-like fibronectin-binding protein of <i>E. faecalis</i>			<i>efbA</i>	EF1249	TTAGCGAGATACGCAACGGT	AAGTCCCAAAAGGAGCCACC	104.61
Enterococcal surface protein			<i>esp</i>	EF0056	GGAACGCCTTGGTATGCTAAC <sup>54</sup>	GCCACTTTATCAGCCTGAACC	No Amp <sup>§</sup>

Gene target		Primer Sequence (5'→ 3')			Efficiency
Name	Symbol*	Locus tag	Forward <sup>†</sup>	Reverse	
FATTY ACID BIOSYNTHESIS					
3-oxoacyl-ACP synthase	<i>fabF-I</i>	EF0283	GCAGGGATTACACCGGAACA	ATGCTCGCTCACCAAAAGACA	No Amp <sup>§</sup>
Enoyl-ACP reductase	<i>fabI</i>	EF0282	GCTTCGCCAGGTGTCAAGAA	ATGCACCGTTAAAAGCAAAGCA	98.47
(3R)-hydroxymyristoyl-ACP dehydratase	<i>fabZ-I</i>	EF0284	AGTGTCCCTGGTGATGTCT	TCCACGTAAGCAGTCGCTTT	102.62
NITROREDUCTASES					
Nitroreductase	EF0404	EF0404	CCATGAGCGAGGAGAGGAGA	GCTAATTGTTGGTCCCGCAC	103.26
Nitroreductase	EF0648	EF0648	CGATGCAACAACCAAGCAGTT	TCCGTCACTCGTTCCCTTGTGC	109.16
Nitroreductase	EF0655	EF0655	TGGTCTGAACAAGCAACTGGT	TGTTGTAGGTTTGGCGCCGAT	109.02
Nitroreductase	EF1181	EF1181	CTTGATCCTGCTTGAGCTGC	TCACCGTTCGTTTCGTCAAT	107.14
REGULATORY FUNCTIONS					
ArgR family transcriptional regulator	<i>ahrC</i>	EF0983	GGCGGCGTGTGGTAGTTTA	CTGCTGCTTCCTCAGTTCGT	98.18
<i>E. faecalis</i> regulator, response regulator	<i>fsr-A</i>	EF1822	GCAACCTCAAAATCCTGCCTG	CACCAGGACGCCCAACTAAA	96.03
<i>E. faecalis</i> regulator	<i>fsr-B</i>	EF1821	GACCTTGGATGACGAGACCG	CGTGCTCAAAAAGCAAAGCC	94.40
<i>E. faecalis</i> regulator, histidine kinase	<i>fsrC</i>	EF1820	ATGTTGAGTGACCGCTCCTT	ATCCAGAAAGAGCACGGTTGG	87.38
Global regulator of virulence, DNA-binding response regulator	<i>grvR</i>	EF1050	CGTTGTCTTGGCACGTGATG	GTAGCCAGTTCCACGGACAG	93.87
Global regulator of virulence, sensor histidine kinase	<i>grvS</i>	EF1051	TGGGGGAAAGACGATCCTGA	CGCGTGAAAGGTCAAAGCAAT	102.44
RNase J2	<i>mjbB</i>	EF1185	TGTCTGGTCACGCTAAATCCG	ACTGGCGGTATTCACCTTGG	102.12
MarR family transcriptional regulator	<i>shyA</i>	EF3002	AGCCTTGTTCTTCCAAACGC	AGGGTCTGTGAAAAATCCTGG G	103.32

Gene target			Primer Sequence (5'→ 3')		Efficiency
Name	Symbol*	Locus tag	Forward <sup>†</sup>	Reverse	
<b>SECRETED FACTORS</b>					
Cytolysin structural subunit, large	<i>cyL<sub>L</sub></i>	EF0525	AGTAGTGGAGATGTTCAAGG	CAGCCACAAGCAGCACTA	No Amp <sup>§</sup>
Cytolysin structural subunit, small	<i>cyL<sub>S</sub></i>	EF0526	TGTTGGTCCTTCTTTTGAAGAGTT	TGCTGGAGTTGCTCAGCCT	No Amp <sup>§</sup>
Gelatinase	<i>gelE</i>	EF1818	GCTCCGATTCCAGCAGAGTT	AGTGATGCTCGTGATGCGAT	97.09
Secreted lipase	<i>salB</i>	EF0394	GCAACTGAATCAAGCACGCA	TCGCTTGGTGTGTAGGTTGT	93.19
<b>STRESS AND DRUG RESPONSE</b>					
Enterococcal MDR efflux	<i>emeA</i>	EF1078	TTTCTCGTGGTTTAGGCGGG	CATCGCTTTCGGACGTTTCAG	102.82
General stress protein 24	<i>gls24</i>	EF0079	CGGTTTGTTAACTGTGGACGG	GACCTCGGTGTGAATGCCTG	105.33
General stress protein B	<i>glsB</i>	EF0080	CAGTCGATGGCGGCTTTTTC	ATCAATACCCGCTGTGACGT	99.04
Organic hydroperoxide resistance protein	<i>ohr</i>	EF0453	GCTTCGCCAGGTGTCAAGAA	ATGCACCGTTAAAGCAAAGCA	95.37
Drug resistance transporter (MFS)	EF0420	EF0420	AATGGAAGGCAGATTGCGCA	AGTCCTTACTGTGCGGCCA	101.26
DNA repair exonuclease	EF0972	EF0972	AACCAAGCAGTACAAGGAG	GCTGCCGACTTTTACCGTACT	109.73
Drug resistance transporter (MFS)	EF1370	EF1370	GGCGGATTGTGCTCTTTGTG	AGTGCAATTTCCCAAGCCCAT	97.49
Macrolide and streptogramin B resistance, protein C (MsrC)-like	EF1413	EF1413	AATGGTGTCTGGGAAGAGTACC	ACGTTCCCTAACCCGGACTTTGG	84.38
ABC transporter ATP-binding protein/peptidase (MDR family)	EF1732	EF1732	GCCCCGCTAGAATAAACTCGCT	GGCTGCGGAATTTGTTTCAGG	100.54
ABC transporter ATP-binding protein/peptidase (MDR family)	EF1733	EF1733	TGCCAACAAATCCCCCAAGGTT	TCAAAACAATGGCGCAAGGG	104.35

\*Symbol to be used as gene reference throughout this thesis; given as common gene symbol when available, otherwise given as locus tag.

<sup>†</sup>Previously validated primers are referenced within the table.

<sup>‡</sup>Locus tag information not available.

<sup>§</sup>Primers did not amplify against *E. faecalis* 1131 genome.

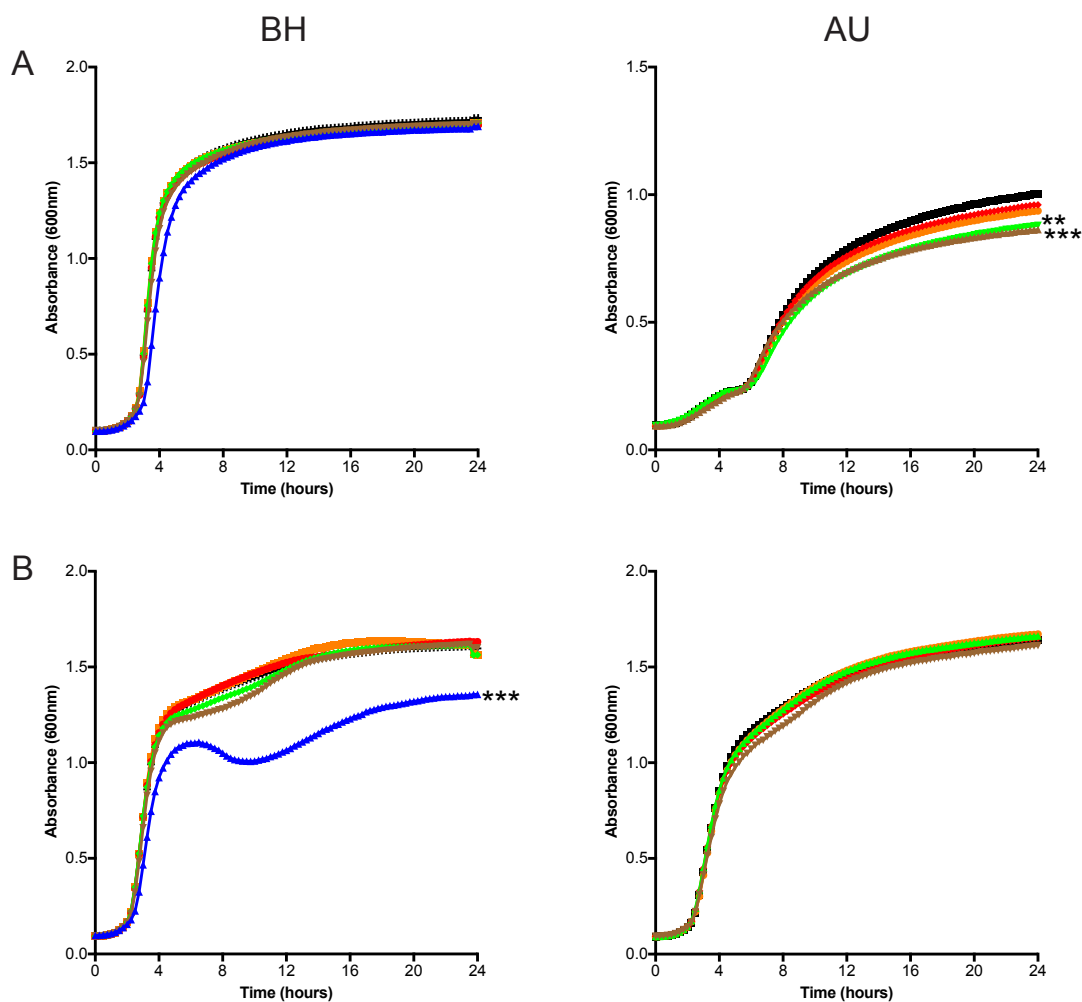
Abbreviations: ACP, acyl carrier protein; DNA, deoxyribonucleic acid; MarR, multiple antibiotic resistance; MDR, multidrug-resistance; MFS, major facilitator superfamily; PavA, adherence and virulence protein A; RNA, ribonucleic acid.

## 2.3 Results

### 2.3.1 Effects of IBU and CIBU on *E. faecalis* growth

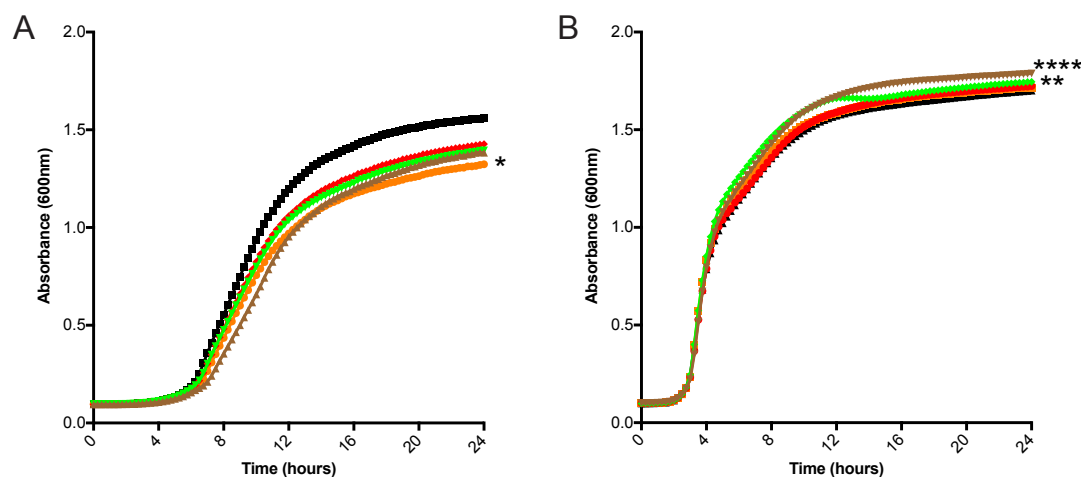
The growth of *E. faecalis* 1396 and *E. coli* CFT073 was assessed in BHI broth in the presence of IBU at 200, 20, 0.2, or 0.02 µg/mL, and in AU with IBU at 20, 0.2, or 0.02 µg/mL. We were unable to assess higher concentrations of IBU in AU given solubility issues. Representative growth curves are presented in Figure 2-1. There was a statistically significant decrease noted in the final optical density (OD) of *E. faecalis* exposed to IBU at 20 and 2 µg/mL ( $P = 0.0006$ ,  $P = 0.002$ ) in AU and *E. coli* at 200 µg/mL IBU in BH ( $P = 0.001$ ). Otherwise, growth patterns were indiscriminate with the exception of a disruption in the absorbance of *E. coli* exposed to 200 µg/mL IBU in BHI.

The effect of CIBU exposure on the growth of *E. faecalis* 1396 was assessed in BH and LB for *E. coli* UTI89 (Figure 2-2). Growth patterns were not significantly altered by the addition of CIBU. However, the final OD of *E. faecalis* was significantly decreased by 0.025 µg/mL CIBU ( $P = 0.03$ ), while the OD of *E. coli* increased significantly by 25 and 2.5 µg/mL CIBU ( $P = 0.0001$ ,  $P = 0.005$ ).



**Figure 2-1. Growth of *E. faecalis* and *E. coli* in the presence of IBU.**

Growth curves are represented as the increase in absorbance over the course of 24 hours. *E. faecalis* 1396 (**A**) and *E. coli* CFT073 (**B**) were grown in BH (left) or AU (right) with vehicle (black) or IBU at 200 (blue), 20 (brown), 2 (green), 0.2 (red), or 0.02 µg/mL (orange). Experiments were conducted as both technical and biological triplicates; data is representative of the average absorbance reading. Absorbance readings were completed every 15 minutes for 24 hours at 600 nm. Significance of the final optical density was determined using one-way ANOVA with Dunnett's multiple comparisons test relative to the vehicle (\*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ).



**Figure 2-2. Growth of *E. faecalis* and *E. coli* in the presence of CIBU.**

Growth curves are represented as the increase in absorbance over the course of 24 hours. *E. faecalis* 1396 was grown in BH (A) and *E. coli* UTI89 in LB (B) with vehicle (black) or CIBU at 25 (brown) 2 (green), 0.25 (red), or 0.025 µg/mL (orange). Experiments were conducted as both technical and biological triplicates; data is representative of the average absorbance reading. Absorbance readings were completed every 15 minutes for 24 hours at 600 nm. Significance of the final optical density was determined using one-way ANOVA with Dunnett's multiple comparisons test relative to the vehicle (\*\*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).

## 2.3.2 Bacterial attachment and invasion

Prior to bacterial attachment and invasion experiments, the MIC of nitrofurantoin and TMP/SMX was determined for *E. faecalis* 1131 and *E. coli* UTI89 (Table 2-3).

### 2.3.2.1 Bacterial attachment in the presence of sub-MIC antibiotics

The attachment of *E. faecalis* 1131 and *E. coli* UTI89 to urothelial cells was determined in the presence of nitrofurantoin and TMP/SMX at the MIC and sub-MICs. There was a statistically significant increase in the attachment of both *E. faecalis* 1131 ( $P = 0.0001$ ) and *E. coli* UTI89 ( $P = 0.005$ ) to the 5637s in response to MIC nitrofurantoin, but not for TMP/SMX (Figure 2-3). There was also a dose-dependent correlation between nitrofurantoin concentration and *E. faecalis* 1131 attachment.

To elucidate whether the increased attachment noted by *E. faecalis* 1131 was related to nitrofurantoin interactions with the urothelial cells, we repeated the experiments with *E. faecalis* under constant antibiotic pressure and pre-treatment only, assessing both attachment and internalisation (Figure 2-4). Enterococcal attachment was significantly increased by nitrofurantoin at the MIC in both the constant pressure ( $P = 0.001$ ) and pre-treatment only ( $P = 0.005$ ) experiments relative to the vehicle control. Further, internalisation was increased by nitrofurantoin at the MIC in the constant antibiotic pressure group ( $P = 0.002$ ), but not in the pre-treatment only group.

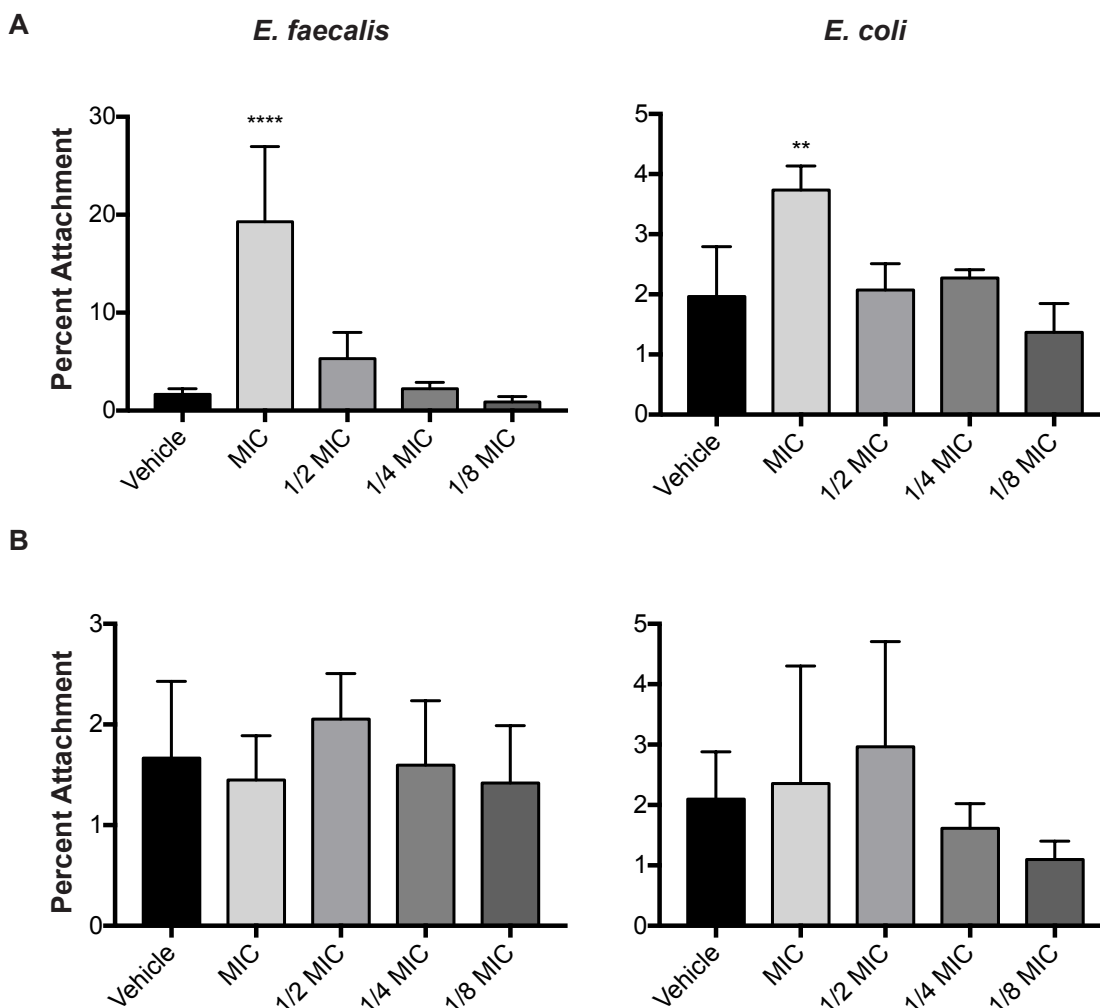


**Table 2-3. Summary of strain MICs.**

Strain	Minimal inhibitory concentration (µg/mL)*		
	Nitrofurantoin	TMP/SMX <sup>†</sup>	IBU
<i>E. faecalis</i> 1131	32	0.07/1.2	≥ 200
<i>E. coli</i> UTI89	8	0.07/1.2	≥ 200

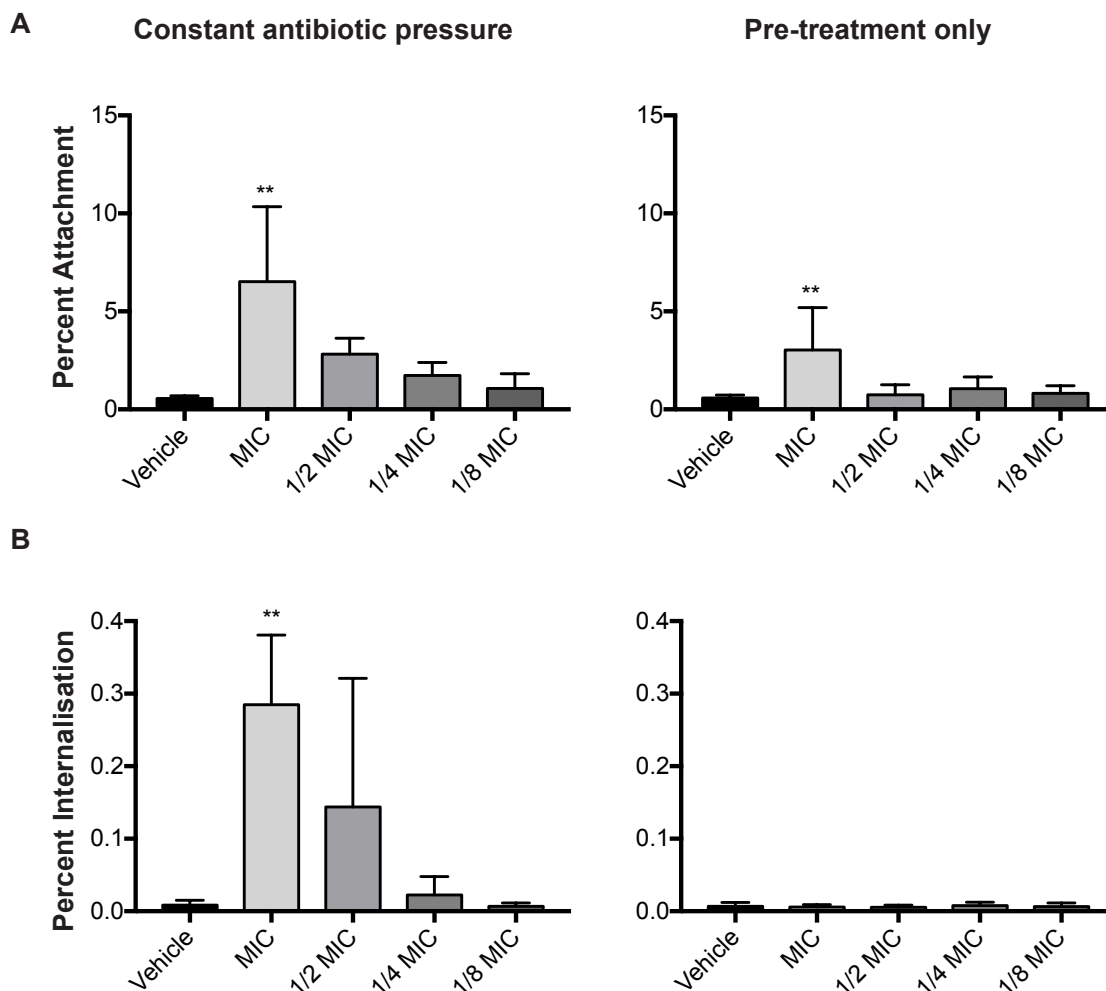
\*Susceptibility profile based on CLSI breakpoints<sup>44</sup>.

<sup>†</sup>*In vitro* activity of TMP/SMX against *E. faecalis* is not an indication of clinical efficacy<sup>44</sup>.



**Figure 2-3. Bacterial attachment to urothelial cells in the presence of antibiotics alone.**

Bacterial attachment to urothelial cells is presented as a percentage of the total viable population. *E. faecalis* 1131 or *E. coli* UTI89 were incubated in the presence of nitrofurantoin (**A**) or TMP/SMX (**B**) at the MIC,  $\frac{1}{2}$  MIC,  $\frac{1}{4}$  MIC, or  $\frac{1}{8}$  MIC then added to a urothelial monolayer in fresh antibiotic-supplemented infection media. Co-cultures were incubated for 4-hours, at which point the cultures were washed with PBS, lysed and bacterial counts acquired by serial dilutions to agar plates. Means from at least three different experiments are shown with significance. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (\*\*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.01$ ).



**Figure 2-4. Nitrofurantoin induced enterococcal attachment and internalisation.**

*E. faecalis* 1131 was exposed to nitrofurantoin at the MIC,  $\frac{1}{2}$  MIC,  $\frac{1}{4}$  MIC, or  $\frac{1}{8}$  MIC then co-cultured with urothelial cells with (constant antibiotic pressure; left) or without (pre-treatment only; right) nitrofurantoin. Percent attachment (**A**) and internalisation (**B**) is presented as a percentage of the total viable population. Gentamicin protection assays were used for internalisation determination, adherence counts were determined by serial dilutions of lysed urothelial cells following a 4-hour co-incubation. Means from at least three different experiments are shown with significance. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (\*\*,  $P < 0.01$ ).

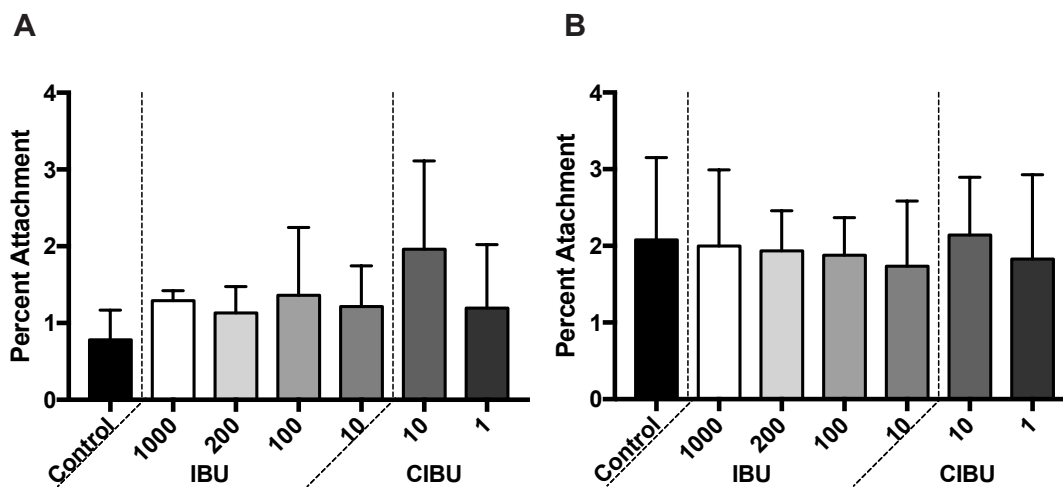
### 2.3.2.2 Bacterial attachment in the presence of IBU and CIBU

We next tested bacterial attachment to urothelial cells in the presence of IBU at 1000, 200, 100, or 10  $\mu\text{g/mL}$ , or CIBU at 10 or 1  $\mu\text{g/mL}$  (Figure 2-5). Vehicle controls, methanol for IBU and DMSO for CIBU, were included; however, as the vehicle did not alter bacterial attachment, the data is not shown. There were no significant differences in bacterial attachment to bladder cells at these concentrations.

### 2.3.2.3 Bacterial attachment in the presence of antibiotics IBU or CIBU

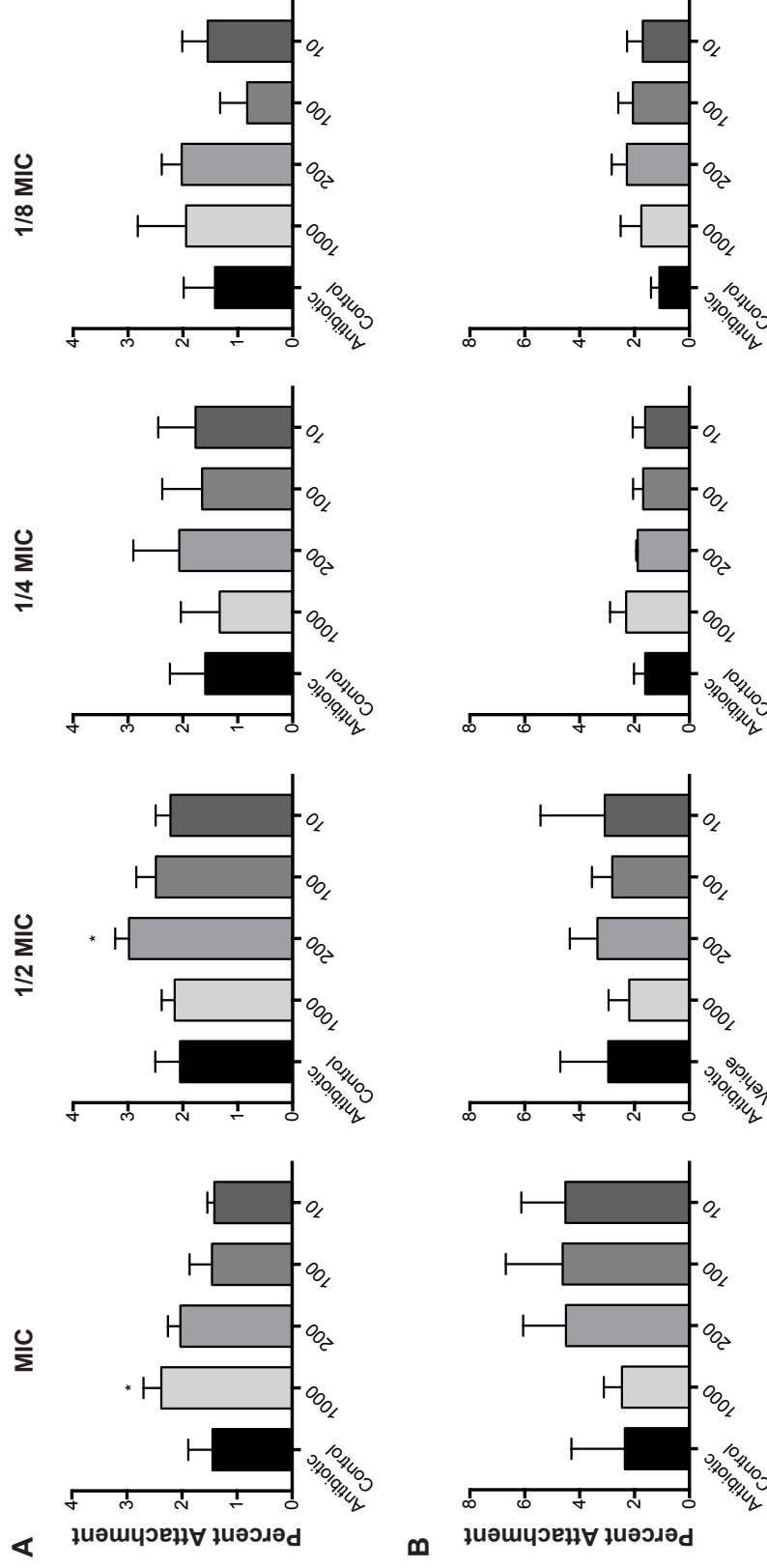
We then assessed the bacterial attachment to urothelial cells in the presence of IBU and either nitrofurantoin or TMP/SMX. Bacteria were pretreated with antibiotic, then brought into contact with urothelial cells with both the antibiotic and IBU. No differences were noted in the attachment of *E. coli* in the presence of IBU and TMP/SMX (Figure 2-6). However, there was a significant increase in *E. faecalis* 1131 attachment at the MIC of TMP/SMX with 1000  $\mu\text{g/mL}$  IBU ( $P = 0.02$ ) and at  $1/2$  MIC with 200  $\mu\text{g/mL}$  IBU ( $P = 0.02$ ). In addition, a positive correlation is visible between IBU concentration and enterococcal attachment at the MIC of TMP/SMX, yet this did not meet statistical significance. IBU did not affect the attachment of *E. faecalis* or *E. coli* to urothelial cells in the presence of nitrofurantoin (Figure 2-7).

Finally, we assayed the attachment of both *E. faecalis* 1131 and *E. coli* UTI89 to urothelial cells in the presence of nitrofurantoin and CIBU (Figure 2-8). Again, there were no significant differences in bacterial attachment related to CIBU exposure.



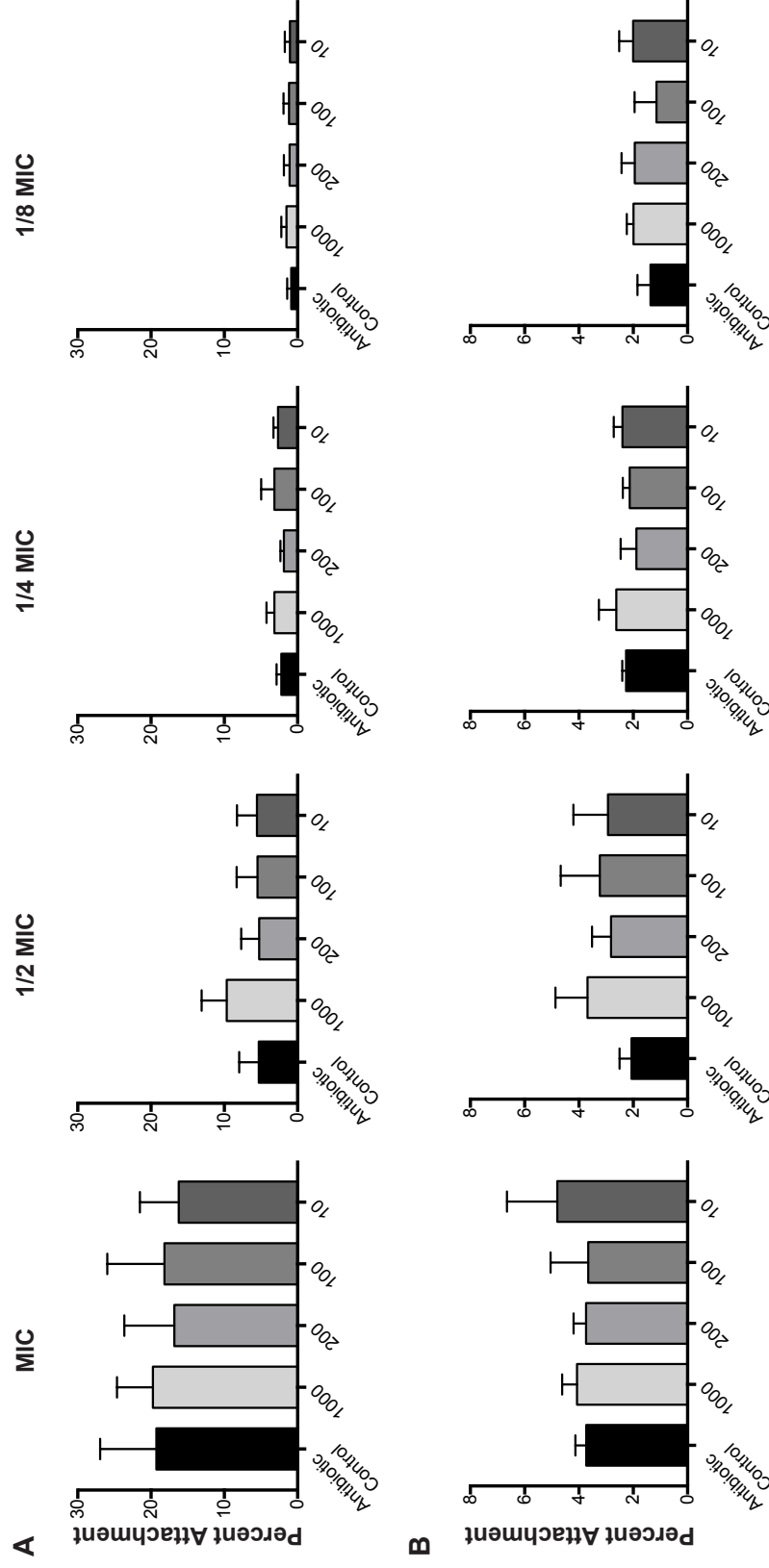
**Figure 2-5. Effects of IBU and CIBU on bacterial attachment.**

The percent attachment of *E. faecalis* 1131 (**A**) and *E. coli* UTI89 (**B**) to urothelial cells was assessed in the presence of 1000, 200, 100, or 10  $\mu\text{g/mL}$  IBU and 10 or 1  $\mu\text{g/mL}$  CIBU. Co-cultures were incubated for 4-hours, at which point the cultures were washed with PBS, lysed and bacterial counts acquired by serial dilutions to agar plates. Means from at least three independent experiments are shown with significance. Lack of significance was confirmed using one-way ANOVA with Dunnett's multiple comparisons test.



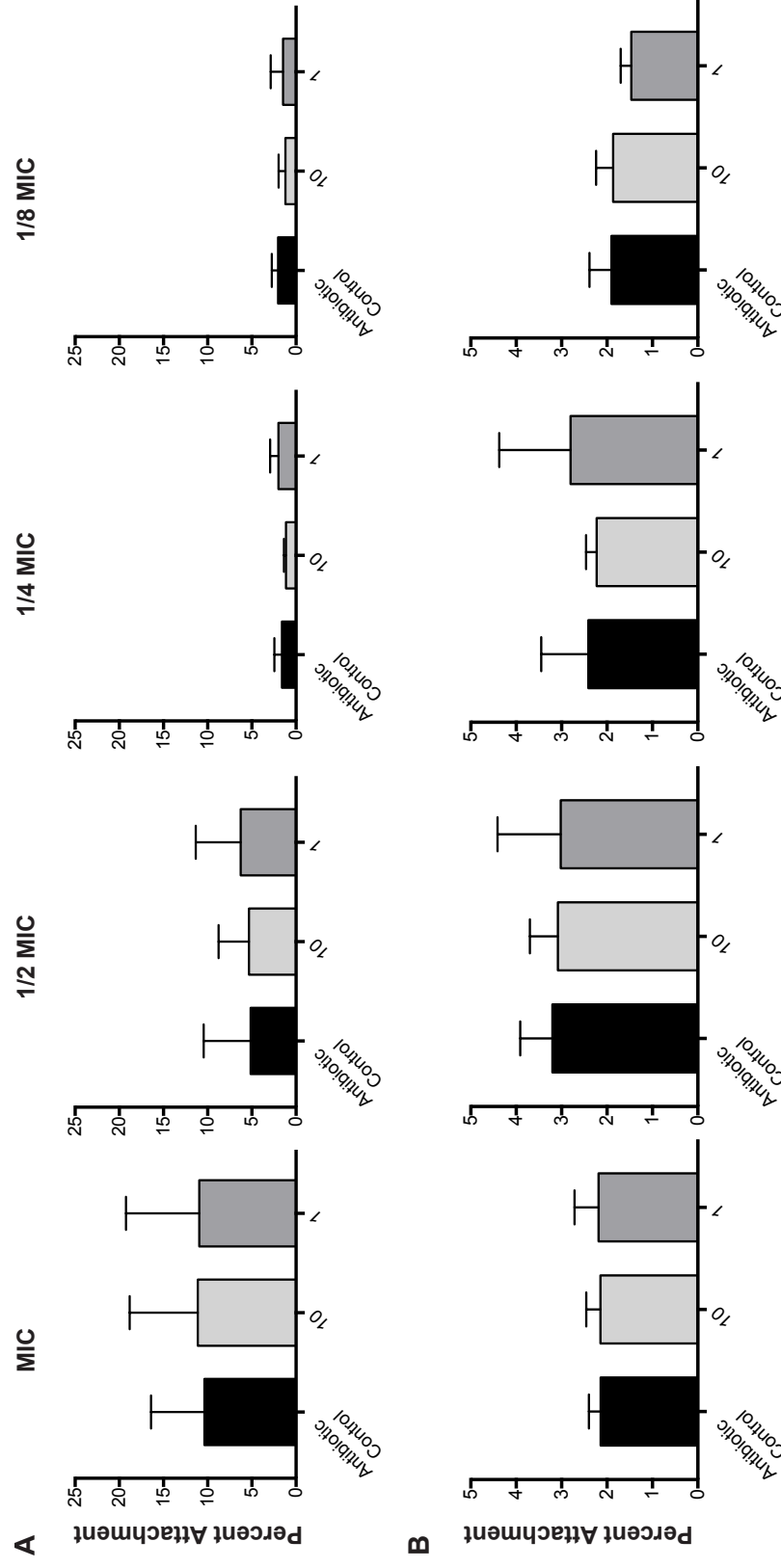
**Figure 2-6. Bacterial attachment to urothelial cells in the presence of TMP/SMX and IBU.**

Bacterial attachment to urothelial cells is presented as a percentage of the total viable population. *E. faecalis* 1131 (A) or *E. coli* UTI89 (B) were incubated in the presence of TMP/SMX at the MIC,  $\frac{1}{2}$  MIC,  $\frac{1}{4}$  MIC, or  $\frac{1}{8}$  MIC then added to a urothelial monolayer in fresh infection media supplemented with the same antibiotic concentration and either vehicle or IBU at concentrations of 1000, 200, 100, or 10  $\mu\text{g/mL}$ . Co-cultures were incubated for 4-hours, at which point the cultures were washed with PBS, lysed and bacterial counts acquired by serial dilutions to agar plates. Means from at least three different experiments are shown with significance. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test (\*,  $P < 0.05$ ).



**Figure 2-7. Bacterial attachment to urothelial cells in the presence of nitrofurantoin and IBU.**

Bacterial attachment to urothelial cells is presented as a percentage of the total viable population. *E. faecalis* 1131 (A) or *E. coli* UT189 (B) were incubated in the presence of nitrofurantoin at the MIC,  $\frac{1}{2}$  MIC,  $\frac{1}{4}$  MIC, or  $\frac{1}{8}$  MIC then added to a urothelial monolayer in fresh infection media supplemented with the same antibiotic concentration and vehicle or IBU at concentrations of 1000, 200, 100, or 10  $\mu\text{g/mL}$ . Co-cultures were incubated for 4-hours, at which point the cultures were washed with PBS, lysed and bacterial counts acquired by serial dilutions to agar plates. Means from at least three different experiments are shown with significance. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test.



**Figure 2-8. Bacterial attachment in the presence of nitrofurantoin and CIBU.**

Bacterial attachment to urothelial cells is presented as a percentage of the total viable population. *E. faecalis* 1131 (**A**) or *E. coli* UT189 (**B**) were incubated in the presence of nitrofurantoin at the MIC,  $\frac{1}{2}$  MIC,  $\frac{1}{4}$  MIC, or  $\frac{1}{8}$  MIC then added to a urothelial monolayer in fresh infection media supplemented with the same antibiotic concentration and vehicle or CIBU at either 10 or 1  $\mu\text{g/mL}$ . Co-cultures were incubated for 4-hours, at which point the cultures were washed with PBS, lysed and bacterial counts acquired by serial dilutions to agar plates Means from at least three different experiments are shown with significance. Significance was determined using one-way ANOVA with Dunnett's multiple comparisons test.



### 2.3.3 Distribution of target genes in *E. faecalis*

Due to the major alterations in *E. faecalis* attachment during antibiotic exposure, isolates of urinary and non-urinary origin were screened for the presence or absence of 37 genes associated with virulence, adherence, host-colonisation, regulation and response to stress (Figure 2-9). Positive PCR reactions were noted for all gene targets against at least one strain except *fabF-1* and *cyLL<sub>S</sub>*. Most strains were positive for all classical virulence factors; however, *asaI* was not present in *E. faecalis* 1131, OG1X or ATCC 19433; *E. faecalis* A256, 1131, OG1X and ATCC 29212 were *esp*<sup>-</sup>; and all but *E. faecalis* 5024-8, ATCC 29212 and 33186 were *cyLL<sub>L</sub>*<sup>-</sup>. Five strains were negative for at least one of the *fsr*-quorum sensing system genes, with *E. faecalis* 5024-8 being negative for all three. *E. faecalis* 5024-8 was also *gelE*<sup>-</sup> and *emeA*<sup>-</sup>.

		Urinary					Non-urinary			
		A256	1131	1396	23241	29212	OG1X	5024-8	33186	19433
Reference	23S									
	recA									
Adhesion and colonisation	ace									
	asa1									
	ebpA									
	ebpB									
	ebpC									
	efaA									
	efbA									
	esp									
Fatty acid biosynthesis	fabF-1									
	fabI									
	fabZ-1									
Nitroreductases	EF0404									
	EF0648									
	EF0655									
	EF1181									
Regulatory functions	ahrC									
	fsrA									
	fsrB									
	fsrC									
	grvR									
	grvS									
	rnjB									
	slyA									
Secreted factors	cylL-L									
	cylL-S									
	gelE									
	salB									
Stress and drug response	emeA									
	gls24									
	glsB									
	ohr									
	EF0420									
	EF0972									
	EF1370									
	EF1413									
	EF1732									
	EF1733									

**Figure 2-9. Distribution of target genes in *E. faecalis* strains.**

Presence or absence of target genes was determined by PCR of *E. faecalis* isolates of both urinary and non-urinary origins. Products of the expected size were considered positive and are represented here by blue boxes, white boxes indicate a failure to amplify.

### 2.3.4 Bacterial gene expression

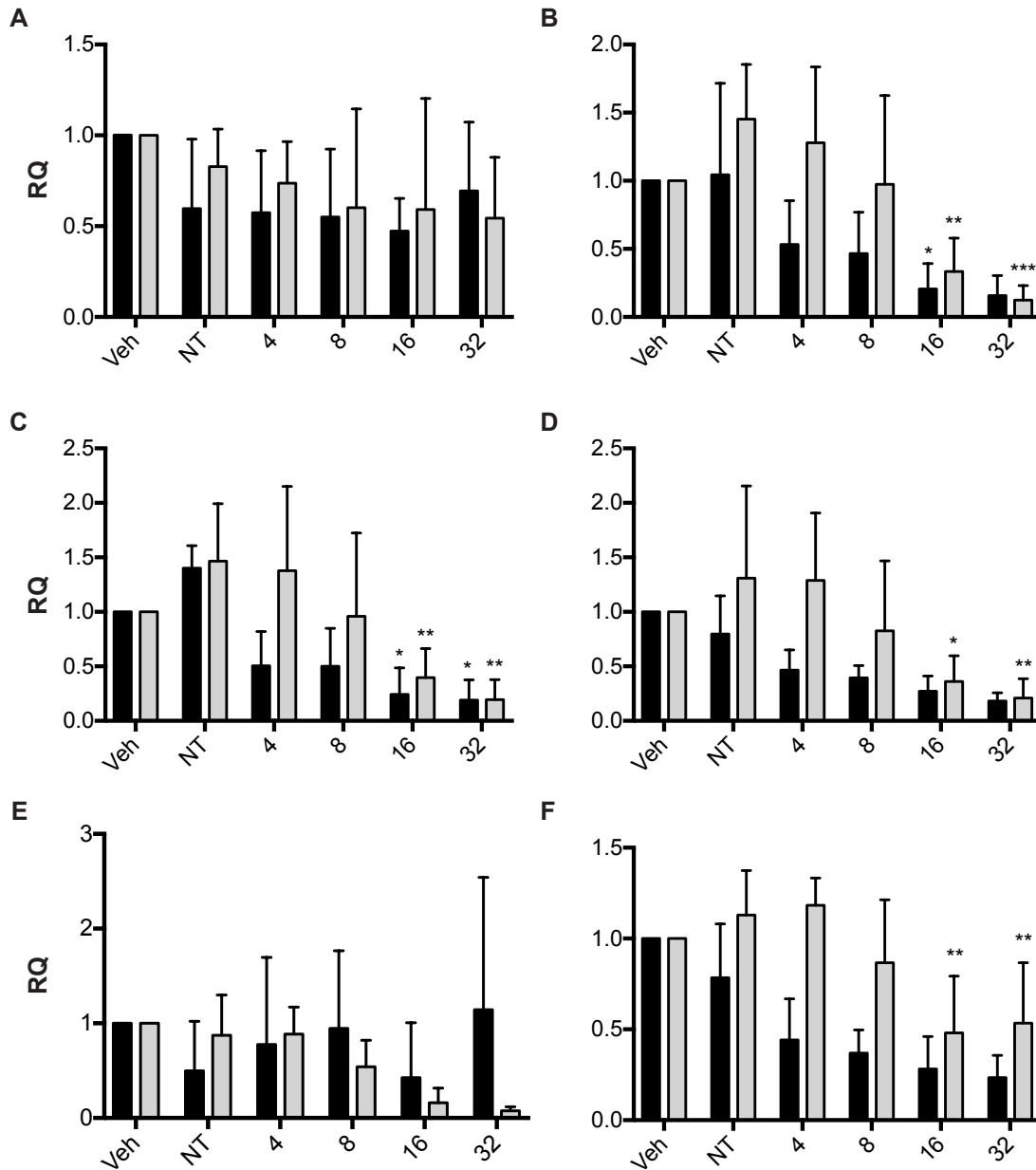
Target gene expression was determined using RNA from *E. faecalis* 1131 co-incubated with urothelial cells for 1 or 4 hours in the presence of nitrofurantoin or vehicle. The PCR screen indicated primers for *fabF-1*, *cylL<sub>S</sub>*, *asa1*, *esp*, and *cylL<sub>L</sub>* would not amplify against *E. faecalis* 1131; thus these targets were not included in the qPCR reactions. Further, reactions for *gelE*, *salB*, *slyA*, and EF1413 were inconsistent; these targets were excluded from the data analysis.

Among the classical *E. faecalis* virulence factors are those involved in adhesion and host colonization, such as *ace*, *ebpABC*, *efaA*, and *efbA* (Figure 2-10). Exposure to nitrofurantoin did not significantly alter expression of *ace* or *efaA*. However, *ebpA* expression was significantly decreased at the MIC (4 hours;  $P = 0.0001$ ) and  $1/2$  MIC (1 hour,  $P = 0.04$ ; 4 hours,  $P = 0.001$ ). Similarly, *ebpB* expression was decreased by 1 hour at both the MIC ( $P = 0.03$ ), and  $1/2$  MIC ( $P = 0.03$ ), and remained so at 4 hours (MIC,  $P = 0.001$ ;  $1/2$  MIC,  $P = 0.006$ ). Expression of *epbC* and *efbA* was decreased only after 4 hours by both  $1/2$  MIC ( $P = 0.01$ ,  $P = 0.05$ ) and MIC ( $P = 0.003$ ,  $P = 0.005$ ).

In contrast, expression of the nitroreductases was generally increased in response to nitrofurantoin (Figure 2-11). Significant increases were observed by the MIC at locus EF0404 (1 hour,  $P = 0.003$ ; 4 hours,  $P = 0.0001$ ), EF0648 (4 hours,  $P = 0.01$ ), and EF0655 (1 hour,  $P = 0.0001$ ; 4 hours,  $P = 0.0001$ ). Increased expression was also noted for EF0655 by  $1/2$  MIC (1 hour,  $P = 0.008$ ), while EF1181 was not significantly differentially regulated. Increased relative expression was also noted for EF1732 and EF1733 of the multidrug-resistance (MDR) family proteins. EF1733 was significantly upregulated at both time points (1 hour,  $P = 0.03$ ; 4 hours,  $P = 0.0001$ ), while EF1732 was significantly upregulated at 4 hours only ( $P = 0.003$ ) (Figure 2-12).

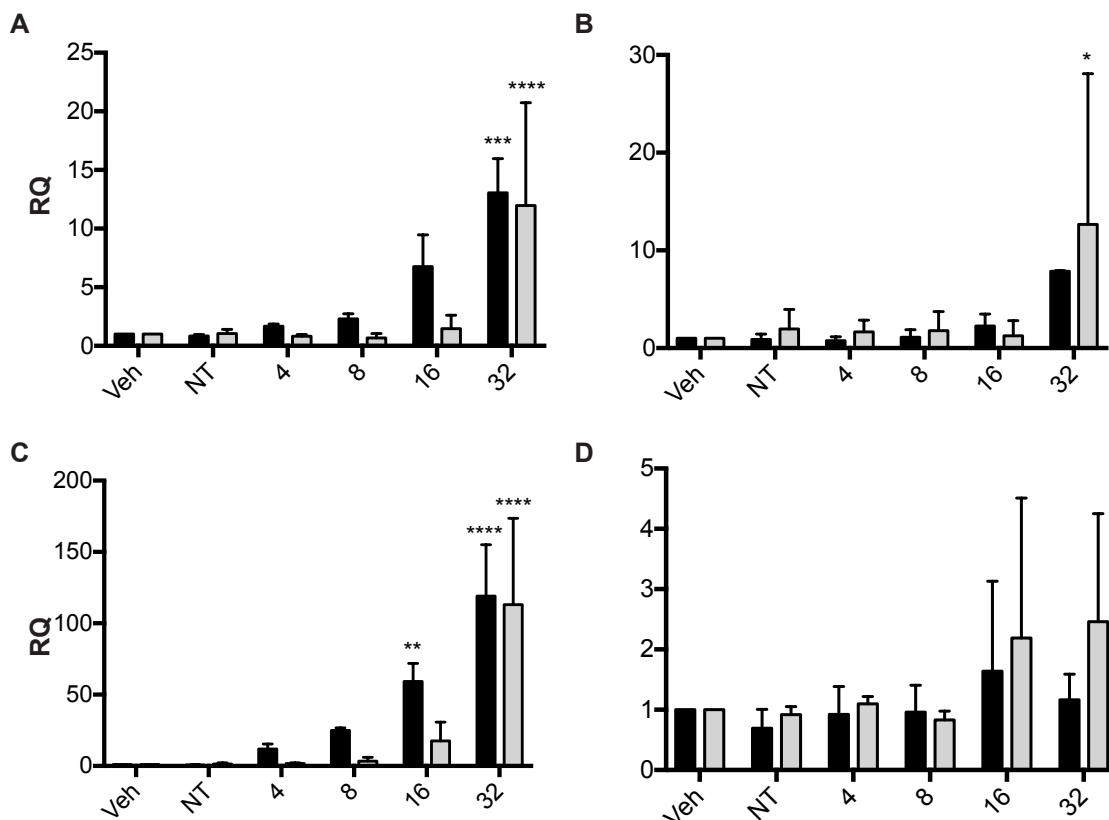
Of the genes involved in stress tolerance (Figure 2-13), EF0972 was differentially expressed in response to nitrofurantoin at the MIC (4 hours;  $P = 0.05$ ) and  $1/2$  MIC (4 hours;  $P = 0.02$ ). The remaining stress tolerance genes were not significantly differentially regulated.

Regulatory genes trended towards decreased expression in response to nitrofurantoin (Figure 2-14). The MIC of nitrofurantoin led to down-regulation of *ahrC* expression (4 hours,  $P = 0.01$ ) and *rnjB* (1 hour,  $P = 0.02$ ; 4 hours,  $P = 0.008$ ). Significant decreases in expression were also noted at  $1/2$  MIC for *ahrC* (4 hours,  $P = 0.02$ ) and *rnjB* (1 hour,  $P = 0.02$ ; 4 hours,  $P = 0.02$ ). There were no significant differences in the expression of *fsrA*, *fsrB*, *grvR*, or *grvS* induced by nitrofurantoin exposure compared to the no treatment controls. Finally, we screened two genes involved in fatty acid biosynthesis, *fabI* and *fabZ-I*; however, differential expression was only noted for *fabZ-I*, which was down-regulated by the MIC at 1 hour ( $P = 0.03$ ; Figure 2-15).



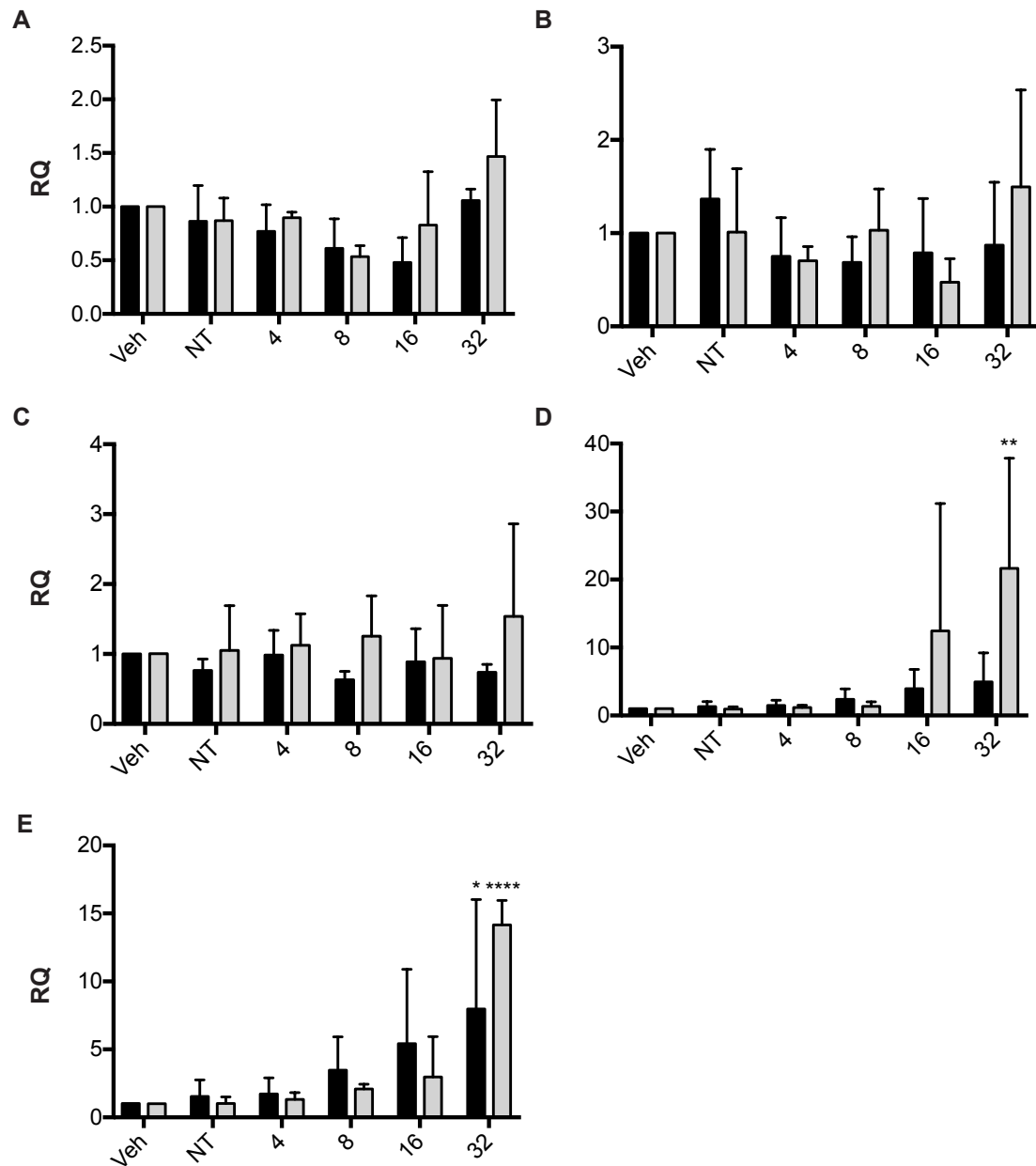
**Figure 2-10. Expression of adhesion and colonisation-associated factors in response to nitrofurantoin.**

Enterococcal gene expression of adhesion and colonisation-associated factors in response to nitrofurantoin challenge in the presence of urothelial cells is presented relative to vehicle controls. Expression was assessed after *E. faecalis* 1131- 5637 co-incubation for 1 hour (black bars) or 4 hours (gray bars) with nitrofurantoin at the MIC (32 µg/mL),  $1/2$  MIC (16 µg/mL),  $1/4$  MIC (8 µg/mL), or  $1/8$  MIC (4 µg/mL) of *ace* (A), *ebpA* (B), *ebpC* (C), *ebpB* (D), *efaA* (E), and *efbA* (F). Experiments and qPCR reactions were completed in triplicate. Means from at least three different experiments are shown with significance. Significance was determined against the no treatment control using two-way ANOVA with Dunnett's multiple comparisons test (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).



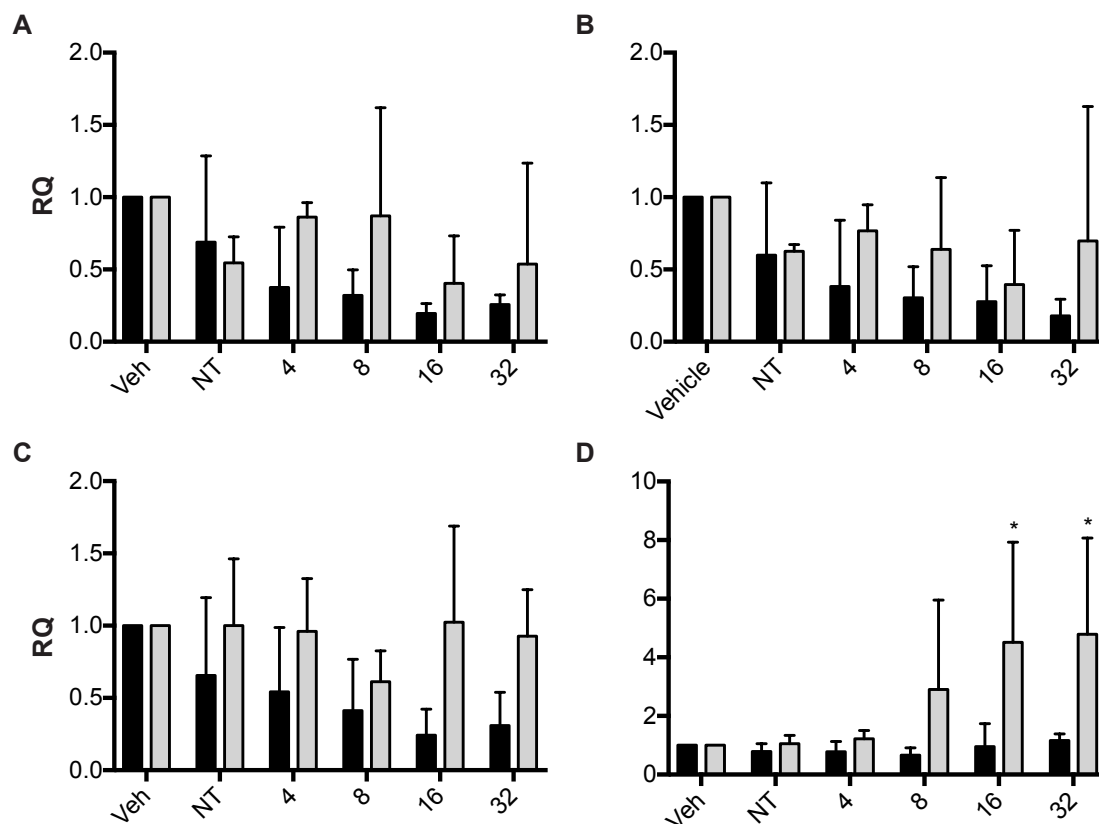
**Figure 2-11. Upregulation of nitroreductase genes in response to nitrofurantoin.**

Enterococcal gene expression of the putative nitroreductases genes; EF0404 (A), EF0648 (B), EF0655 (C), and EF1181 (D), in response to nitrofurantoin challenge is presented relative to vehicle controls. Expression was assessed after *E. faecalis* 1131- 5637 co-incubation for 1 hour (black bars) or 4 hours (gray bars) with nitrofurantoin at the MIC (32 µg/mL),  $\frac{1}{2}$  MIC (16 µg/mL),  $\frac{1}{4}$  MIC (8 µg/mL), or  $\frac{1}{8}$  MIC (4 µg/mL). Experiments and qPCR reactions were completed in triplicate. Means from at least three different experiments are shown with significance. Significance was determined against the no treatment control using two-way ANOVA with Dunnett's multiple comparisons test (\*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).



**Figure 2-12. Relative expression of MDR genes to nitrofurantoin exposure.**

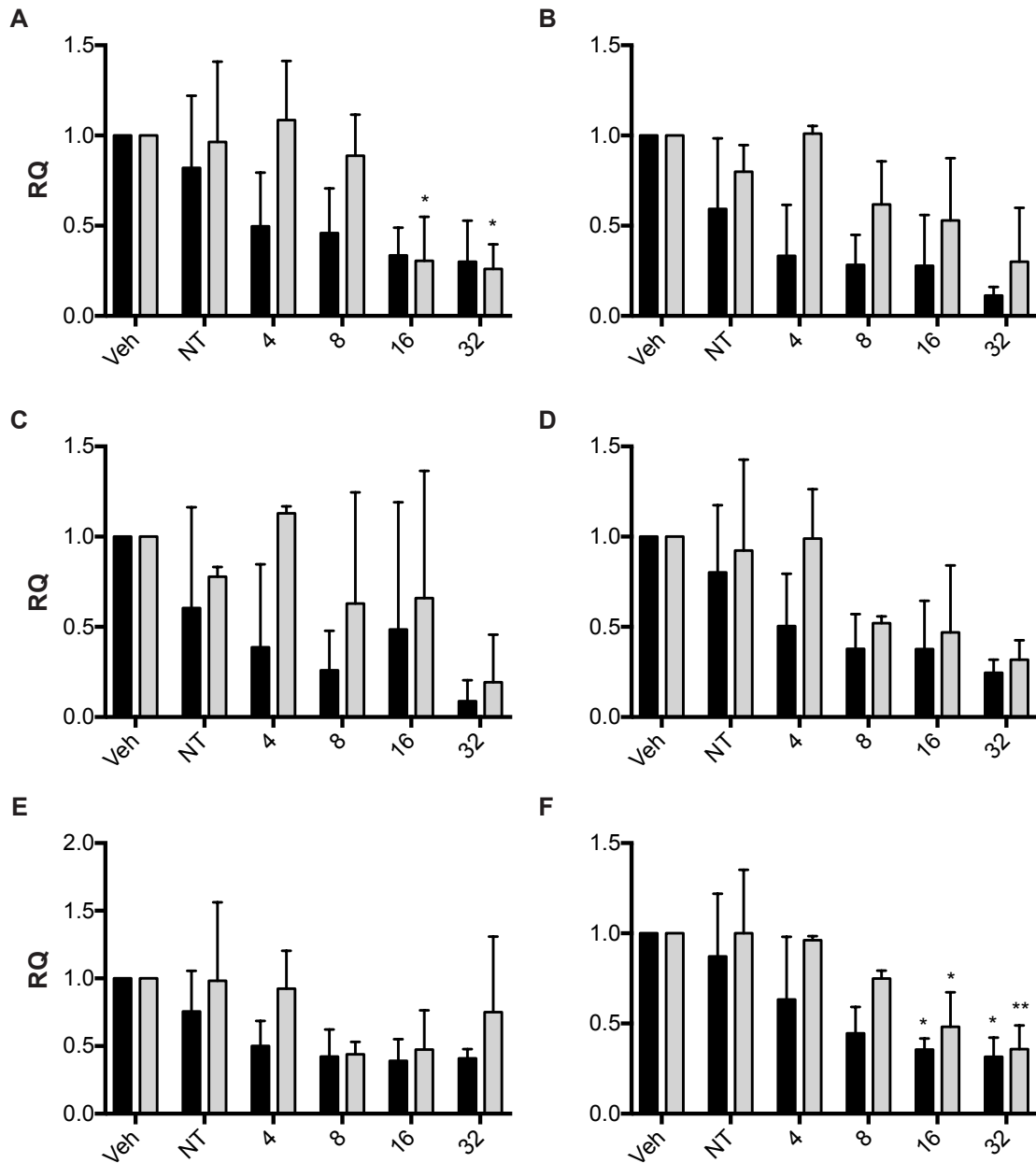
Enterococcal gene expression of MDR genes in response to nitrofurantoin challenge is presented relative to vehicle controls. Expression of *emeA* (A), EF420 (B), EF1370 (C), EF1732 (D), and EF1733 (E) was assessed after *E. faecalis* 1131- 5637 co-incubation for 1 hour (black bars) or 4 hours (gray bars) with nitrofurantoin at the MIC (32 µg/mL),  $1/2$  MIC (16 µg/mL),  $1/4$  MIC (8 µg/mL), or  $1/8$  MIC (4 µg/mL). Experiments and qPCR reactions were completed in triplicate. Means from at least three different experiments are shown with significance. Significance was determined against the no treatment control using two-way ANOVA with Dunnett's multiple comparisons test (\*\*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).



**Figure 2-13. Relative expression of enterococcal stress response genes following nitrofurantoin exposure.**

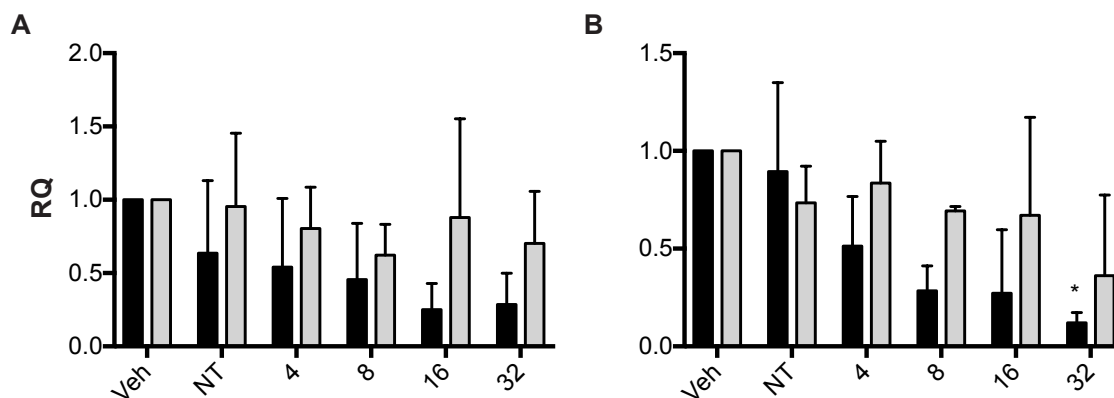
Enterococcal gene expression following nitrofurantoin exposure of genes involved in the stress response is presented relative to vehicle. Expression of *gls24* (A), *glsB* (B), *ohr* (C), and EF0972 (D) was assessed after *E. faecalis* 1131- 5637 co-incubation for 1 hour (black bars) or 4 hours (gray bars) with nitrofurantoin at the MIC (32 µg/mL),  $1/2$  MIC (16 µg/mL),  $1/4$  MIC (8 µg/mL), or  $1/8$  MIC (4 µg/mL). Experiments and qPCR reactions were completed in triplicate. Means from at least three different experiments are shown with significance. Significance was determined against the no treatment control using two-way ANOVA with Dunnett's multiple comparisons test (\*,  $P < 0.05$ ).





**Figure 2-14. Expression of enterococcal regulatory genes in response to nitrofurantoin.**

Enterococcal gene expression of regulatory genes in response to nitrofurantoin challenge is presented relative to vehicle controls. Expression of *ahrC* (A), *fsrA* (B), *fsrB* (C), *grvR* (D), *grvS* (E), *rnjB* (F) was assessed after *E. faecalis* 1131-5637 co-incubation for 1 hour (black bars) or 4 hours (gray bars) with nitrofurantoin at the MIC (32 µg/mL),  $1/2$  MIC (16 µg/mL),  $1/4$  MIC (8 µg/mL), or  $1/8$  MIC (4 µg/mL). Experiments and qPCR reactions were completed in triplicate. Means from at least three different experiments are shown with significance. Significance was determined against the no treatment control using two-way ANOVA with Dunnett's multiple comparisons test (\*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ).



**Figure 2-15. Differential expression of fatty acid biosynthesis genes in response to nitrofurantoin.**

Enterococcal gene expression of the fatty acid biosynthesis genes, *fabI* (A) and *fabZ-1* (B) in response to nitrofurantoin challenge at the MIC (32 µg/mL),  $\frac{1}{2}$  MIC (16 µg/mL),  $\frac{1}{4}$  MIC (8 µg/mL), or  $\frac{1}{8}$  MIC (4 µg/mL) in the presence of urothelial cells (black bars, 1 hour; gray bars, 4 hours). Expression is presented as relative to the vehicle. Experiments and qPCR reactions were completed in triplicate. Means from at least three different experiments are shown with significance. Significance was determined against the no treatment control using two-way ANOVA with Dunnett's multiple comparisons test (\*,  $P < 0.05$ ).

## 2.4 Discussion

In this chapter, we describe nitrofurantoin-induced attachment of *E. faecalis* and *E. coli* to urothelial cells, and characterisation of the enterococcal response to nitrofurantoin using qPCR. Additionally, we explored *in vitro* effects of IBU and its metabolite, CIBU, alone and with antibiotics for the reduction of bacterial attachment.

Co-culture experiments were completed with 5637s and either *E. faecalis* 1131 or *E. coli* UTI89. Flow chamber experiments with *E. faecalis* 1131 demonstrated increased adherence to silicone in the presence of  $1/8$  MIC of vancomycin relative to control, but not  $1/4$  MIC or sub-MICs of ampicillin<sup>59</sup>, suggesting this strain differentially responds to antibiotics at sub-MICs. *E. coli* UTI89 is a classically studied UPEC and served as a Gram-negative comparator.

An unexpected finding of our co-culture experiments was the significant increase in attachment of both *E. faecalis* and *E. coli* to urothelial cells in response to nitrofurantoin at the MIC (Figure 2-3). This was contrary to the expected trend, namely increased attachment at sub-MICs, as has been noted for *S. saprophyticus* and *E. coli* in response to ciprofloxacin, ampicillin, and gentamicin<sup>15,16</sup>. The lack of response to various concentrations of TMP/SMX is also noteworthy; our data suggests, that, at least in the case of *E. coli*, TMP/SMX may be an effective prophylactic agent. Unfortunately, the same cannot be said for its action against *E. faecalis*, as TMP/SMX is not considered clinically active against this bacterium. However, our data does suggest administration of TMP/SMX for enterococcal UTI is not likely to cause harm through an inadvertent increase in enterococcal adherence and colonisation.

To the best of our knowledge, this is the first time increased attachment has been documented for nitrofurantoin above sub-inhibitory concentrations. Previous reports have remarked on the variable effects of  $1/4$  MIC nitrofurantoin on the attachment of *E. coli*, wherein approximately half of strains increased and half decreased attachment in response to the antibiotic<sup>60</sup>. Notably,  $1/4$  and  $1/2$  MIC TMP/SMX also demonstrated variable effects on *E. coli* attachment, yet we did not observe this for *E. coli* UTI89 or *E. faecalis* 1131.

In addition to bacterial interactions, nitrofurantoin is cytotoxic to mammalian cells and has been implicated in oxidative stress, inducing single-strand DNA breaks<sup>61,62</sup>. Thus, we sought to identify if the increase in bacterial attachment was a result of the urothelial cell's cytotoxic response. To do so, we repeated attachment experiments with two general groups, pre-treatment only and constant pressure (Figure 2-4). The increase in bacterial attachment remained significant in the pre-treatment only group, suggesting it was not due to urothelial cytotoxicity induced by nitrofurantoin. Further, internalisation was increased by nitrofurantoin at the MIC in the constant pressure group, but not the pre-treatment only group. Thus, the nitrofurantoin appears to be inducing a selective pressure on the *E. faecalis*.

Given this major increase in bacterial attachment, we chose to assess the transcriptional response of *E. faecalis* to nitrofurantoin. We developed a panel of target genes that are differentially regulated in human pooled urine or in response to antibiotic stress<sup>51-54</sup>. Our panel also included nitroreductases and known virulence factors (Table 2-2).

We first assessed the prevalence of these genes in our *E. faecalis* collection through a conventional PCR screen. Many groups have studied the genetic distribution of virulence factors in clinical and environmental isolates of *E. faecalis*<sup>58,63-65</sup>; however, a large proportion of our strain collection is not widely available, nor do they have publicly deposited genomes. Additionally, *E. faecalis* ATCC 29212 and 33186 are primarily used as quality control strains in antibiotic susceptibility testing; thus genetic study of these strains is not commonplace. Until quite recently<sup>66</sup>, no research had been published on the presence or activity of nitroreductases in *E. faecalis*, although their presence has been assumed given sensitivity to nitrofurans. Our data demonstrating increased adherence in the presence of nitrofurantoin MICs suggest an understanding of nitroreductases in *E. faecalis* may present future antibiotic targets.

Unsurprisingly, each of the four nitroreductases genes, EF0404, EF0648, EF0655, and EF1181, were conserved across the strains, as were genes involved in fatty acid biosynthesis and stress and drug responsive factors (Figure 2-9). Conservation was also noted for regulatory genes, except for the *fsrABC* locus. Five of our strains were negative

for at least one of the genes in the *fsr*-quorum sensing system. This correlates with the high incidence of *fsrABC* deletions in *E. faecalis*, which has been noted by multiple groups. In one study of uropathogenic *E. faecalis*, 79% of isolates negative for gelatinase activity had deletions in the *fsrABC* region, yet were *gelE-sprE*<sup>+67</sup>. Another noted 50% of urine isolates were gelatinase negative, and 44% of these were *fsrABC*<sup>-63</sup>. A screen of Greek clinical samples noted only 21% of isolates were *fsrB*<sup>+65</sup>. Most groups attribute the loss of *fsrABC* to the same 23.9-kbp deletion, which has been noted in almost 60% of *fsrABC* clinical isolates<sup>68</sup>, and may explain the loss of *fsrABC* in *E. faecalis* 5024-8. Loss of the *fsr*-system is noteworthy as the system regulates virulence factor expression, including that of the *ebp* locus, *gelE*, extracellular serine protease (*sprE*), and *efaA*<sup>69-71</sup>.

*E. faecalis* 5024-8 was the only strain to test negative for *gelE* and *emeA*. A plethora of information exists regarding the distribution and function of *gelE* in disease pathogenesis; however, minimal information is available on *emeA*. Indeed, since its initial discovery in 2001<sup>72</sup>, a total of six papers have been published on this gene<sup>33,72-76</sup>. Initially characterised as an MDR efflux pump of the major facilitator superfamily (MFS)<sup>72</sup>, EmeA, has since been linked to increased fluoroquinolone and ethidium bromide resistance<sup>33,72,75</sup>. Further studies of gene distribution suggest ciprofloxacin, gatifloxacin, and levofloxacin resistant *E. faecalis* are significantly more likely to be *emeA*<sup>+</sup> than antibiotic sensitive strains<sup>33</sup>. In contrast, the general prevalence of *emeA* in *E. faecalis* varies depending on the report. A screen of *E. faecalis* from human, animal, and food sources noted 90% of isolates were *emeA*<sup>+74</sup>, yet analysis of Chinese clinical isolates indicated only 26% of *E. faecalis* are *emeA*<sup>+33</sup>. Most recently, 63% of *E. faecalis* from a swine meat line were positive<sup>73</sup>. Our data confirm *E. faecalis* 29212 as *emeA*<sup>+</sup> and support a high prevalence of *emeA* in clinical isolates, particularly those associated with the urinary tract<sup>75</sup>.

In agreement with larger-scale studies, *efaA* and *ace* were noted in all of our samples<sup>64,77</sup>. Findings with regards to *asaI* (66%), *gelE* (89%), and *esp* (56%) were also comparable to clinical isolates from the University of Freiburg (80, 100, and 60% respectively)<sup>64</sup>. Other groups have reported *esp* in 40-70% of human isolates<sup>65,77</sup>. AsaI is one of the best-studied aggregation substance (AS) proteins, which are encoded on pheromone-inducible

conjugative plasmids<sup>78</sup>. Aggregation substance, and Asa1 in particular is involved in bacterial conjugation<sup>79</sup>, adherence to epithelial cells via interaction with the extracellular matrix<sup>80</sup>, and adherence to and survival within macrophages<sup>81</sup>. Similarly, Esp promotes primary attachment and biofilm formation by *E. faecalis*<sup>82</sup>, as well as colonisation and persistence within the lower urinary tract<sup>83</sup>. GelE, a secreted zinc metalloprotease is also involved in biofilm formation<sup>78</sup>. Expression of *gelE* is dependent on activation of the fsr-quorum sensing system, and is responsible for cleavage of AltA, a peptidoglycan hydrolase key to daughter cell separation, and SprE, among other targets<sup>84,85</sup>.

There were no positive reactions for generated for *fabF-1* or *cylL<sub>S</sub>*, although *in silico* analysis indicated a positive reaction for *fabF-1* against the genome of *E. faecalis* 29212. There are a number of reasons these negative reactions may have occurred, including inappropriate reaction conditions, interference of secondary structures within the primers, or lack of binding sites on the template – indeed, these apply to all of our negative reactions. Given the wide selection of other targets, such as two additional fatty acid biosynthesis genes (*fabF-1* and *fabZ-1*) and the primarily negative response to *cylL<sub>L</sub>*, these targets were dismissed from further analysis.

Given the high prevalence of our gene targets, we moved forward with measuring the relative expression of these genes in response to nitrofurantoin. The PCR screen indicated primers for *fabF-1*, *cylL<sub>S</sub>*, *asa1*, *esp*, and *cylL<sub>L</sub>* would not amplify against *E. faecalis* 1131; thus these targets were not included in the qPCR reactions. Further, reactions for *gelE*, *salB*, *slyA* and EF1413 were inconsistent; these targets were not included in the data analysis.

Expression of genes involved in adhesion and colonisation highlighted significant downregulation of *ebpA*, *ebpB*, *ebpC* and *efbA* (Figure 2-10). Expression of *efaA* trends towards decreased expression associated with nitrofurantoin concentration, yet this was not significant. These results were unexpected, as we noted an increase in bacterial attachment at the MIC, yet the expression of genes involved in the early stages of bacterial biofilm formation were down-regulated. Notably, the Ebp pilus is associated with primary adherence and biofilm formation<sup>71</sup>. Further, *efbA* and *ebpABC* mutants are

severely attenuated in both adherence and UTI pathogenesis<sup>86,87</sup>. Down-regulation was also noted for *fabZ-1*, yet this was only significant at the MIC and one hour. Expression of *fabI* also trends toward a concentration-dependent decrease in expression at one hour, yet this was not significant (Figure 2-15). Interestingly, *fabI* and *fabZ-1* are up-regulated in urine, potentially as a contributor to cell membrane remodelling<sup>51</sup>, which was also somewhat expected in response to nitrofurantoin.

Down-regulation was also noted for the regulatory genes, although this was only significant for *ahrC* and *rnjB*. There were no significant differences in the relative expression of *fsrA*, *fsrB*, *grvR*, or *grvS*; however, these did trend towards decreased expression. A transcription factor, AhrC, is involved in biofilm formation and is necessary for enterococcal endocarditis and UTI<sup>88</sup>. Similarly, RNase J2, the product of *rnjB* has a regulatory role. Knockout of *rnjB* correlated with decreased expression of *ebpABC*, *gls24*, *glsB*, *gelE*, *fsrC*, and *fsrB* *in vitro*, suggesting RNase J2 plays a role in regulating biological cues for fitness and virulence<sup>89</sup>. This is consistent with our data, as the enterococci appear to be responding to the nitrofurantoin through RNase J2, given that there was a significant decrease in the relative expression of *ebpABC*, and a trend in down-regulation of *gls24*, *glsB*, *fsrB*, and *fsrC* at 1 hour.

The MDR efflux pumps encoded by the loci EF1732 and EF1733 were significantly up-regulated, suggesting the enterococcal response to nitrofurantoin includes removal of the compound or its breakdown products from the cell. These loci are also upregulated by *E. faecalis* in response to chloramphenicol and erythromycin<sup>52,53</sup>.

In line with these results, the nitroreductases EF0404, EF0648, and EF0655 were significantly up-regulated by nitrofurantoin at the MIC (Figure 2-12). Increased relative expression was also noted for EF1181, yet this did not meet statistical significance. This is particularly significant, as nitroreductases were only officially documented in *E. faecalis* last year<sup>66</sup>. Indeed, our work supports these findings and highlights that each is likely differentially regulated and responsive to nitrofurantoin. Further, transcriptomic studies of *E. faecalis* in human pooled urine showed an increase in the expression of EF0655 relative to culture media<sup>51</sup>.

Of the stress response genes assayed, increased relative expression was noted for locus EF0972 at 4 hours, while the expression of *gls24*, *glsB*, and *ohr* decreases slightly with increasing MIC, yet this lacked statistical significance. However, *gls24*, *glsB*, and *ohr* were significantly repressed in response to erythromycin and chloramphenicol and up-regulated in human pooled urine<sup>51-53</sup>. The EF0972 locus encodes a putative DNA repair exonuclease, thus increased expression of this gene target is expected, given DNA damage is a mechanism of nitrofurantoin action. Further, an increase in expression of EF0972 was also noted in *E. faecalis* exposed to erythromycin<sup>53</sup>, suggesting EF0972 may be involved in the enterococcal response to multiple antibiotic classes.

Given these major increases in bacterial attachment associated with antibiotics, we moved on to characterisation of the antimicrobial properties of IBU against *E. faecalis* and *E. coli*. Early clinical and animal studies suggested that NSAIDs, and in particular IBU, may have a place in the treatment of UTI, as an alternative to antibiotics. This is not yet widely accepted, given that antibiotics cure the infection and IBU is only regarded as symptom relief. Ibuprofen is widely available, relatively cheap, associated with minimal adverse reactions, and has an established safety profile<sup>90</sup>. With the global increases in antibiotic resistance, lack of financial incentive for antibiotic development and subsequent lack of new antibiotic classes in the development pipeline, many researchers have begun to explore the off-label use of approved drugs, including IBU. If IBU exhibits antimicrobial properties it may assist the patient's immune system in clearing the infection. Further, Given the high spontaneous resolution rate associated with UTI, the option to reduce discomfort and thereby avoid antibiotic prescription, IBU represents a mechanism to reduce the burden of antibiotic side effects, resistance, and cost.

Humans metabolise ibuprofen to two major metabolites, 2-hydroxyibuprofen and 2-carboxyibuprofen (CIBU)<sup>30</sup>. Excretion of IBU, CIBU, and 2-hydroxyibuprofen occurs as both the free and glucuronic acid conjugated forms in the urine. Other metabolites are found at much lower concentrations in the urine, including 1-hydroxyibuprofen and 3-hydroxyibuprofen<sup>91</sup>. Data on urinary excretion of IBU metabolites varies; patients receiving a long-term dose excreted 8% as 2-hydroxyibuprofen (free and conjugated forms) and 28% as CIBU (free and conjugated forms)<sup>92</sup>. While healthy males were found



to excrete 26% as 2-hydroxyibuprofen (free and conjugated forms), 42% as CIBU (free and conjugated forms), and 12% as conjugated IBU<sup>93</sup>. Other studies suggest 10-15% is excreted as unmodified IBU, 25-30% as 2-hydroxyibuprofen, and 30-40% as CIBU<sup>91</sup>. The classical view is that the hydroxy and carboxy metabolites are biologically inactive<sup>94</sup>; current literature fails to identify a link between these metabolites and pharmacological activity.

We assessed the ability of IBU and its major metabolite, CIBU to alter the growth kinetics of *E. faecalis* and *E. coli* in rich bacterial culture media (BHI or LB) and AU. Under our experimental conditions, neither IBU nor CIBU were bactericidal (Figure 2-1 and 2-2). There were significant decreases in the final OD of *E. faecalis* exposed to 20 and 2 µg/mL IBU in AU and *E. coli* exposed to 200 µg/mL in BHI; however, whether these are physiologically relevant decreases is debatable. Indeed, we can likely attribute decrease in *E. coli* OD to bacterial clumping. In contrast, the effects of CIBU on the growth of *E. faecalis* and *E. coli* was variable and deserves further investigation, particularly in the context of human urine as the BHI and LB used for these experiments are not representative of the urinary environment.

Accordingly, other groups have documented the MIC of IBU to be 5000 µM, or 1000 µg/mL, for *S. aureus*, *Bacillus subtilis*, and *Acinetobacter baylyi*, yet the MIC for *E. coli* was out of the assay range<sup>95</sup>. In contrast, the same study identified significantly lower MICs for other NSAIDs, such vedaprofen, bromfenac, and carprofen, especially for the Gram-positives and this was linked to inhibition of DNA replication<sup>95</sup>. Other reports document MICs of 150 µg/mL IBU for *S. aureus*, and 50 µg/mL IBU for uropathogenic *E. coli* (UPEC) and *Helicobacter pylori*<sup>28,96,97</sup>. In a murine model of active tuberculosis, oral IBU reduced the bacillary load and increased animal survival, yet IBU is known to be ineffective against *Mycobacterium tuberculosis*, suggesting the antimicrobial effects may be anti-inflammatory mediated<sup>98</sup>.

Ibuprofen is a non-selective inhibitor of COX enzymes, which are responsible for the conversion of arachidonic acid to prostaglandins. Both the constitutively expressed *COX-1* and the inducible *COX-2* are expressed by urothelial cells<sup>99-101</sup>, leading to the urinary

release of prostanoids, such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)<sup>102</sup>. Prostaglandins are known primarily for their contribution to pain and inflammation; hence their production is an effective target for anti-inflammatories<sup>102,103</sup>. Given these downstream effects, it is not surprising that bacterial virulence factors, such as pyocyanin, lipopolysaccharide, and type I fimbriae increase PGE<sub>2</sub> release *in vitro*<sup>100,101</sup>. Urinary PGE<sub>2</sub> is also higher in patients with active UTI relative to healthy controls, and the concentration decreases significantly upon completion of antibiotic therapy<sup>104</sup>. Further, a murine study of UTI suggested that COX-2 inhibition significantly reduces bacterial titers and the incidence of chronic cystitis<sup>25</sup>. These findings, combined with the efficacy of COX-inhibitors in the prevention and treatment of UTI *in vivo*, suggests IBU may disrupt bacterial adherence to the urothelium.

Adhesion studies were completed with IBU and CIBU at concentrations between 1000 and 1 µg/mL to mimic the urinary environment; however, the literature lacks consensus on the concentration of IBU in human urine. For example, two hours after a 400 mg oral dose the urinary concentration of IBU was 153.5 µg/mL in one individual, this figure was 98.3 µg/mL at four hours and 109.44 µg/mL at eight hours, the concentration then proceeded to drop to 16.51 µg/mL by 12 hours and 6.76 µg/mL by 14<sup>30</sup>. In another individual, five hours after oral administration of 600 mg, urinary concentration of IBU was 14.7 µg/mL<sup>49</sup>. Yet another group identified concentrations of 2.5 µg/mL two hours after a 200 mg dose and 1.74 µg/mL after three hours<sup>50</sup>. Finally, urine was collected up to 36 hours after a 200 mg dose in ten participants; urinary recovery ranged from 18.2 to 1,563 ng/mL of IBU and 181.2 to 4,176 ng/mL of CIBU<sup>48</sup>. Thus, while 200, 100, and 10 µg/mL are likely to be encountered by bacteria in the bladder, 1000 µg/mL IBU is not and represents an extreme.

Our attachment results did not indicate either an increase or decrease in the attachment of *E. faecalis* or *E. coli* to urothelial cells in response to IBU alone or in conjunction with TMP/SMX or nitrofurantoin (Figures 2-5, 2-6, 2-7). These experiments were completed with *E. coli* UTI89 and *E. faecalis* 1131; a uropathogenic strain that our lab had previously indicated was capable of adhering to both hydrophobic and hydrophilic surfaces *in vitro*<sup>37,105</sup>. There are two notable exceptions to this trend; attachment was

significantly increased by *E. faecalis* at the MIC of TMP/SMX and 1000 µg/mL IBU and at  $1/2$  MIC with 200 µg/mL IBU. The data also appear to trend towards a positive correlation between IBU concentration and enterococcal attachment, when exposed to TMP/SMX at the MIC. This did not meet statistical significance, yet could represent an adaptive stress response.

Similarly, CIBU did not affect bacterial attachment, regardless of whether the bacteria were pre-treated with nitrofurantoin or not; we did not assess TMP/SMX due to material costs (Figures 2-5 and 2-8). In addition, 10 µg/mL CIBU appeared to be up-regulating attachment, yet this was not statistically significant and may present an experimental artefact. Future experiments should assess interactions of co-cultures with higher concentrations of CIBU; however, its low solubility is a complicating factor. Further, our data questions the status-quo that CIBU is biologically inactive<sup>91</sup>.

In contrast to our findings, other groups have identified ibuprofen as an antimicrobial potentiator, wherein co-administration of IBU with an antimicrobial increased the microbe's sensitivity to the antimicrobial<sup>106</sup>. In general, antibiotic potentiators restore or enhance the clinical utility of antibiotics, extending the spectrum of activity and increasing safety as lower concentrations become effective<sup>107</sup>. Using both an *in vitro* and murine model of *Candida albicans* infection, IBU was found to potentiate the activity of fluconazole and was synergistic in the treatment of murine sepsis with Pep19-2.5, an antimicrobial peptide<sup>108,109</sup>. Similarly, celecoxib, a COX-2 specific inhibitor, increased the sensitivity of MDR *S. aureus* and *Mycobacterium smegmatis* to ampicillin, kanamycin, ciprofloxacin, and chloramphenicol through inhibition of an MDR efflux pump<sup>110</sup>. This widespread effect against fungi and bacteria further supports the potentiator concept.

While intriguing, our attachment data comes with a number of limitations. First of all, the large increase in uropathogen attachment could be a factor of the final concentration of bacteria. The number of bacteria added to the co-incubation was the same for all antibiotic concentrations, yet the MIC inhibited enterococcal growth, while growth continued, albeit at different rates, at  $1/2$ ,  $1/4$ , and  $1/8$  MIC. Therefore, the same number of

bacteria may be attaching to the urothelial cells in each condition, yet as we represent the data as a fraction of the total, the percent attachment may be artificially increased. However, increased attachment was also significant within our pre-treatment group (Figure 2-4), suggesting that inhibition of bacterial growth may exaggerate the observed effect, but is not an indication of experimental artefact. To clarify this, experiments should be repeated with the use of microscopy.

The four-hour time point may have been too early to catch the impact of IBU. In murine studies, *COX-2* is barely detectable in the bladders of uninfected mice, or at 6-hours after UPEC infection; however, by 24-hours post-infection *COX-2* expression is increased 50-fold<sup>25</sup>. Cell culture studies show a similar trend, *COX-2* mRNA expression begins to increase within 30 minutes of infection and peaks at 4 hours; however, PGE<sub>2</sub> release only increases after 8 hours<sup>101</sup>.

A further limitation lies within the cell line itself; 5637s do not display the morphological characteristics of umbrella cells, lack uroplakin (UP) plaques and do not have a glucosaminoglycan (GAG) layer. In a healthy bladder, the GAG layer and UP plaques are believed to impede the ability of bacteria to adhere to the urothelium. In a “remodeled” bladder, the superficial layer of cells lack umbrella cell differentiation factors, including UP plaques. Thus, as a model of RUTI, 5637 are acceptable as they are a better representation of the intermediate cell type bacteria will encounter in the bladder.

Moving forward, our attachment and invasion studies could be repeated with the use of microscopy to confirm the increased attachment and characterise potential morphological changes that may be driving the phenotype. It would also be worth conducting transcriptomics studies to identify new drug targets. Enterococcal invasion of urothelial cells is a relatively recent discovery that warrants detailed characterisation. Our data suggests antibiotics have the potential to increase uropathogens invasion, if this holds true *in vivo* antibiotic prescribing practices for UTI may need to seriously reconsidered.

### 2.4.1 Conclusions

These findings highlight the importance of understanding interactions between bacteria and antibiotics at a range of concentrations, especially when considering antibiotics that are bacteriostatic. Interestingly, the bactericidal properties of nitrofurantoin against enterococci begin after 24 hours at  $16 \times \text{MIC}$ , yet complete killing is still not observed after 24 hours at  $32 \times \text{MIC}$ <sup>111</sup>. Our data suggest enterococci exhibit increased virulence at concentrations of nitrofurantoin that are inhibitory to growth, a finding not previously described. This increased attachment correlates with a relative decrease in expression of adherence factors, regulatory factors, and fatty acid biosynthesis and an increase in expression of nitroreductases, MDR efflux pumps, and a DNA repair exonuclease. We also demonstrated inhibitory concentrations of nitrofurantoin increased the rate of urothelial cell invasion by *Enterococcus*. If this holds true *in vivo*, it may explain the high rates of recurrence encountered after antibiotic prophylaxis.

We also identified three potential new antibiotic targets EF1732, EF1733, and EF0972. Expression of these gene products was previously shown to be increased by erythromycin<sup>53</sup>, and an increase in expression has also been described for EF1732 and EF1733 in response to chloramphenicol<sup>52</sup>. Unfortunately, that is the extent of the research available on these gene loci. However, our results, in combination with the erythromycin and chloramphenicol data suggest target of these proteins in conjunction with antibiotics presents a valid therapeutic option.

On a final note, many researchers have begun to consider IBU a “non-antibiotic,” a compound that displays antimicrobial activity although the primary function is not infection related<sup>106</sup>. Research into this field has led to the formation of categories based on the antimicrobial mechanism of action. Our research indicates IBU does not have direct antimicrobial properties nor potentiate the activity of TMP/SMX or nitrofurantoin *in vitro* against *E. faecalis* or *E. coli*. This contradicts clinical reports and murine models. Given the lack of research characterising the exact mechanism of action, we believe that IBU’s “antimicrobial” properties are a result of its anti-inflammatory effects<sup>26</sup>. Further research into the contribution of PGs to UTI pathogenesis is key to elucidating this mechanism.

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## Chapter 3

### 3 Longitudinal study of the bacterial communities in urine from children with RUTI

#### 3.1 Introduction

Urinary tract infections (UTI) are the second most common cause of community and hospital-acquired infections in children and adults<sup>1-3</sup>. Up to 8% of children will experience a UTI before the age of 7<sup>4-7</sup>, and risk is increased between the ages of 3 and 5<sup>5,8</sup>. One of the greatest challenges associated with acute UTI is the development of recurrent UTI (RUTI), which is defined as three or more symptomatic UTIs in a 12-month period, or two or more within six months. Recurrence rates are particularly high in children; primary care estimates suggest up to 12% of boys and girls per year will recur<sup>5</sup>, while referral and emergency department data indicates 20-48% will recur within 12 months<sup>9,10</sup>.

*Enterococcus faecalis* is one of the most commonly isolated pathogens from patients with both UTI and asymptomatic bacteriuria (ABU)<sup>2,5,11-14</sup>. Many reports list *E. faecalis* as the second most common uropathogen<sup>15-17</sup>, while *E. coli* is universally recognised as the most common, accounting for over 80% of UTIs<sup>18</sup>. Interestingly, UTIs caused by enterococci are significantly less likely to meet the standardised diagnostic criteria relative to other uropathogens<sup>12</sup>.

Diagnosis of UTI is primarily made based on symptomology and the presence of significant bacteriuria, yet the cutoffs for this are widely disputed, have not been formally validated in children, and do not provide a measure of disease severity or renal involvement<sup>4</sup>. This traditional definition of UTI, i.e. invasion of a single pathogen beyond an artificially defined load threshold, has been further questioned by the recent characterisation of the urinary microbiota. Indeed, this definition is based on the assumption that the urinary tract is sterile proximal to the urethra in states of health. The application of 16S rRNA gene sequencing to the urine of healthy and diseased adults

invalidates this assumption and suggests that urinary conditions, including UTI, may be states of dysbiosis<sup>19,20</sup>.

The urinary microbiota of men was first described in 2010<sup>21</sup>, was quickly followed by the female urinary microbiota in 2011<sup>22</sup>, and has since expanded to include over 20 publications, which correlate altered community profiles with various states of disease (Chapter 1, Table 1-1)<sup>19,23-26</sup>. However, all but two of these studies are cross-sectional<sup>27,28</sup>, none have studied a paediatric population, and none of the studies have addressed the impact of antibiotics on the urinary microbiota. Given that antibiotic therapy is the standard of care for UTI<sup>29-31</sup>, an understanding of the microbial response to this intervention is key.

Antibiotic prophylaxis, the long-term low-dose use of antibiotics, is often prescribed for the treatment and prevention of RUTI<sup>4,32</sup>. In children, prophylaxis is primarily recommended as a strategy to reduce the risk of pyelonephritis, renal scarring, and febrile UTI<sup>9</sup>; yet clinical studies do not demonstrate these benefits<sup>4,7,10,33</sup>. Infection recurrence is quite high following antibiotic prophylaxis<sup>34</sup>, which may be linked to the development of persister cells or bacterial invasion of the urothelium<sup>35,36</sup>. Of note, recurrence rates are higher in children receiving antibiotic therapy for enterococcal UTI in comparison to Gram-negative UTIs<sup>37</sup>. Likewise, antibiotic treatment of enterococcal ABU is associated with an increased risk of symptomatic UTI<sup>38</sup>.

Relative to other pathogens, enterococcal pathogenesis within the bladder is severely understudied. Enterococcal adherence to and biofilm formation on urothelial cells has been well documented *in vitro* and in mouse models of UTI<sup>39,40</sup>, yet invasion of the bladder epithelium was only recently reported<sup>41</sup>. Furthermore, Chapter 2 has demonstrated the *in vitro* increase of enterococcal attachment and invasion to urothelial cells by exposure to nitrofurantoin, an antibiotic commonly used in the management of UTI and RUTI.

In this chapter, we describe a longitudinal study of RUTI in paediatric patients receiving antibiotic prophylaxis and those under clinical observation that were not prescribed prophylactic antibiotics. We hypothesised that *Enterococcus faecalis* would be more



prevalent in the urine of paediatric patients with a history of RUTI relative to healthy controls and that antibiotic prophylaxis would increase the incidence of uropathogens within the RUTI population.

## 3.2 Materials and methods

### 3.2.1 Study design and clinical sample collection

Paediatric patients with a history of RUTI were recruited through the urology clinic at Children's Hospital at London Health Sciences Centre (London, ON, CAN; NCT02357758). Ethical approval for the study was granted by the Health Sciences Research Ethics Board at the University of Western Ontario (REB 102817, Appendix B) and Lawson Health Research Institute (CRIC R-12-387, Appendix B), both of London, Ontario, Canada. The methods were carried out according to the approved guidelines; written consent was obtained from the parent/legal guardian at the time of study inclusion, and when appropriate, assent was also provided by the participant (Appendix C).

The study proposed to recruit 75 participants over a 6-month recruitment period, including 20-30 healthy controls, with participants followed over a 12-month period. Inclusion and exclusion criteria for the RUTI group are provided in Table 3-1. All patients were required to have an ultrasound to identify urinary tract abnormalities. In the event of an abnormal ultrasound or a history of greater than two febrile UTIs, patients underwent a voiding cystourethrogram (VCUG), to rule out vesicoureteral reflux (VUR). Patients meeting the inclusion criteria were recruited to the study during regularly scheduled clinic appointments.

Upon recruitment, patients were assessed for bladder and bowel dysfunction (BBD) through completion of uroflowmetry, and review of a 48-hour bowel bladder diary and dysfunctional voiding scoring system questionnaire (DVSS; Appendix C)<sup>42</sup>. As is standard for this clinic, the 48-hour bowel bladder diary was mailed out in the clinic information package and reviewed during the initial consultation.

Recurrent UTI patients were divided into two groups; 1) patients receiving antibiotic prophylaxis, or 2) patients solely undergoing clinical observation, based on the clinical experience of the attending urologist and perceived UTI severity. Antibiotic prophylaxis patients were prescribed either trimethoprim-sulfamethoxazole (TMP/SMX, Septra; trimethoprim at 2 mg/kg) or nitrofurantoin (2 mg/kg) for 6-months, followed by a 6-month washout period. Patients were provided with prescriptions in the clinic, and the antibiotic was acquired in a community pharmacy. Antibiotic compliance was not formally assessed; however, participants were asked to report the time during which they followed the antibiotic regimen. In the event of a breakthrough UTI (a UTI experienced while on antibiotic prophylaxis), the antibiotic was discontinued and the UTI treated with an alternative antibiotic; when appropriate, the prophylactic agent was switched. Patients under clinical observation received antibiotics if they experienced a UTI during the study; these were often treated by the family doctor. Patients under clinical observation and those receiving antibiotic prophylaxis were counselled on UTI prevention strategies including lifestyle changes, behavioural modifications, and constipation management.

Participants provided both first-void (FV) and midstream (MS) urine at the initial appointment (baseline) and at 3-month intervals, up to 12-months, during scheduled appointments. Instructions for urine collection were given to the parent/legal guardian for in-clinic collection. First-void included approximately the first 10 mL, and the remainder was MS urine. The parent/legal guardian collected FV samples as soon as the child started to void, then quickly switched bottles for the MS samples. Urinalysis was completed in the clinic by nursing staff using urinalysis strips. Urine samples were only collected from patients when they were non-symptomatic and kept on ice until processing.

Age-matched, healthy controls were also recruited; however, they were only asked to provide a urine sample at one time-point. Healthy controls had no history of RUTI, no presentation of urinary tract abnormalities, and no antibiotic exposure in the last six months.

### 3.2.2 Urine sample processing

Urine samples underwent bacteriological study using both culture-dependent and independent (16S rRNA gene sequencing) methods, and quantitation of urinary cytokines. In brief, 200  $\mu$ L of urine was used for bacterial culture, two 1.5 mL aliquots saved for cytokine and content analysis, and the remainder stored for 16S rRNA gene sequencing. Storage of urine samples was at -80°C. Given the study time-frame, the standard procedure for extraction of DNA from urine samples varied throughout the study – half of the samples were stored at -80°C as unprocessed urine and half were first centrifuged at room temperature for 10 minutes at  $5,000 \times g$  and the pellet stored at -80°C. Samples stored as unprocessed urine were centrifuged under the same conditions and the pellet used for DNA extraction; to prevent multiple freeze-thaw effects, these samples were pelleted on the day of DNA extraction. Storage conditions were recorded to identify confounding factors in 16S rRNA gene sequencing analysis associated with storage conditions.

Uropathogenic bacteria were enumerated and presumptively identified from first-void and midstream urine using CHROMagar Orientation (BD, Franklin Lakes, NJ, USA) and Columbia Blood Agar (BD) supplemented with 5% defibrinated sheep's blood (CBA; Thermo Fisher Scientific, Waltham, MA, USA). Unprocessed or 10-fold dilutions in phosphate buffered saline (PBS) were spread on agar plates and incubated for 48 hours at 37°C under atmospheric conditions. Pure cultures were added to the Burton-Reid uropathogen collection for future study. Heatmaps of bacterial culture data were prepared using Superheat v0.1.0 (<https://github.com/rlbarter/superheat>) in R v3.4.3 (<https://www.R-project.org>)<sup>43,44</sup>.

**Table 3-1. Inclusion and exclusion criteria for the RUTI group of this study.**

<b>Inclusion criteria</b>	<b>Exclusion criteria</b>
Age: 3-15 years old	Age: < 3 or > 15 years old
Toilet-trained	Not toilet-trained
History of symptomatic, culture-proven RUTI, defined as: <ul style="list-style-type: none"> <li>- two or more infections in the last year, or</li> <li>- more than 3 infections overall.</li> </ul>	No documented history of symptomatic, culture-proven RUTI
Normal renal and bladder ultrasound	Renal or bladder ultrasound demonstrate; <ul style="list-style-type: none"> <li>- signs of injury,</li> <li>- anatomical abnormalities,</li> <li>- blockage or kidney stones,</li> <li>- cysts or tumors,</li> <li>- diverticula,</li> <li>- high volume of post-void residual urine.</li> </ul>
Normal VCUG*	VCUG indicative of VUR
Willing and able to receive antibiotic prophylaxis as Septra or nitrofurantoin, when clinically indicated	Unwilling or unable to receive antibiotic prophylaxis; <ul style="list-style-type: none"> <li>- history of allergic reaction to Septra or nitrofurantoin,</li> <li>- medical comorbidity preventing the use of prophylaxis.</li> </ul>
No clinical evidence of neurogenic bladder	History of neural tube defect, spinal cord injury or disease

\*Patients underwent a VCUG if there was a history of greater than two febrile UTIs or the ultrasound results were abnormal.

Abbreviations: RUTI, recurrent urinary tract infection; UTI, urinary tract infection; VCUG, voiding cystourethrogram; VUR, vesicoureteral reflux.

### 3.2.3 DNA isolation and 16S rRNA sequencing

DNA was isolated from urine samples using the DNeasy PowerSoil HTP 96 Kit according to the manufacturer's instructions (Qiagen, Toronto, ON, CAN). In brief, pellets were suspended in 200  $\mu$ L DNase-, RNase-free water (Qiagen), then transferred to individual wells of the PowerSoil-htp Bead Plate containing 750  $\mu$ L PowerSoil-htp Bead Solution and 60  $\mu$ L Solution C1. Plates were incubated at 65°C for 10 minutes followed by shaking for 20 minutes at speed 20 using the MO BIO 96-well plate shaker (Qiagen). Plates were centrifuged at room temperature for 12 minutes at  $2,250 \times g$ , and 500  $\mu$ L of the supernatant from each well transferred to wells of a fresh plate containing 250  $\mu$ L Solution C2 and mixed by pipetting. The plates were incubated at 4°C for 10 minutes followed by centrifugation at room temperature for 12 minutes at  $2,250 \times g$ . The resulting supernatant was then transferred to individual wells of a fresh plate and the centrifugation step repeated. The resulting solution, approximately 600  $\mu$ L per well, was then transferred to a fresh plate containing 200  $\mu$ L Solution C3 and mixed by pipetting. The plate was incubated at 4°C for 10 minutes followed by centrifugation for 12 minutes at  $2,250 \times g$ . The entire volume, with the exception of the pellet, was again transferred to a fresh plate for centrifugation at room temperature for 12 minutes at  $2,250 \times g$ . Avoiding the residual pellet, 650  $\mu$ L was transferred from each well to a fresh plate containing 650  $\mu$ L Solution C4 followed by an additional 650  $\mu$ L of Solution C4. Samples were mixed by pipetting and 500  $\mu$ L of solution transferred to a Spin Plate, which was centrifuged at room temperature for 7 minutes at  $2,250 \times g$ . The flow-through was discarded, and this step repeated until the entire sample-Solution C4 mix was processed through the Spin Plate. A volume of 500  $\mu$ L Solution C5-D was then added to the Spin Plate. The plate was centrifuged twice at room temperature for 10 minutes at  $2,250 \times g$ ; the flow-through was discarded between spins. Following the second centrifugation, 100  $\mu$ L Solution C6 was added to the Spin Plate, incubated at room temperature for 10 minutes and the resulting DNA eluted to a Microplate via centrifugation at room temperature for 15 minutes at  $2,250 \times g$ . DNA was stored at -20°C until amplified for sequencing.

PCR amplification of 1  $\mu$ L DNA was completed using the Earth Microbiome universal primers, 515F and 806R, which are specific for the V4 variable region of the 16S rRNA

gene<sup>45</sup>. Amplification was carried out using Promega GoTaq hot-start colourless master mix (Promega, Madison WI, USA) for 25 cycles as 1 minute each of 95°C, 52°C, and 72°C, preceded by 3 minutes at 95°C to activate the GoTaq. Inline barcodes were designed as the left- or right-side Illumina adaptor at the 5' end followed by 4 random nucleotides, the 8-nucleotide barcode and the associated left or right primer at the 3' end<sup>46</sup>.

DNA extraction and PCR amplification were completed across a total of four 96-well plates. Urine samples were processed in parallel with samples from other experiments. DNA extraction and no template PCR controls were also included.

Sequencing was carried out on the MiSeq System (Illumina Inc., San Diego, CA, USA) at the London Regional Genomics Centre (<http://www.lrgc.ca>; London, ON, CAN) using the 600-cycle MiSeq Reagent Kit v3 (Illumina Inc.). Paired-end sequencing was carried out as 2 × 220 cycles with the addition of 5% PhiX-174. Data was exported as raw fastq files.

### 3.2.4 Microbial composition analysis

Fastq files were de-multiplexed into forward and reverse reads and non-biological nucleotides removed using an internal pipeline designed and maintained by members of the Gloor lab (<https://github.com/ggloor>). Sequence files were then processed using DADA2 v0.99.7 (<https://github.com/benjjneb/dada2>)<sup>47</sup>. In brief, DADA2 was used to filter and trim the reads, de-replicate, perform sample inference and error rate estimation, merge paired reads, remove chimaeras and assign taxonomy against the SILVA rRNA reference database v123 (<https://www.arb-silva.de>)<sup>48</sup>. The resulting sequence table was used for downstream analyses.

Compositional data analysis was completed with CoDaSeq v0.99.1 (<https://github.com/ggloor/CoDaSeq>) and ALDEx2 v1.10.0 ([https://github.com/ggloor/ALDEx2\\_dev](https://github.com/ggloor/ALDEx2_dev)) in R<sup>43,49-53</sup>. Use of compositional data tools is important, as it takes into account that high-throughput sequencing systems have a fixed capacity. Thus, sequencing results provide a random sample of the underlying bacterial

ecosystem, which cannot be related to the total number of bacteria in the input sample. Other methods of 16S rRNA gene sequencing analysis, such as rarefaction and differential abundance, lead to loss of information and unacceptably high false positive rates<sup>46</sup>. Principle component analysis (PCA) plots were prepared using CoDaSeq according to the following; each sample had a minimum of 1000 reads, and each amplicon sequence variants (ASVs) had to make up a minimum of 0.1% of all reads. Samples or ASVs not meeting these criteria were removed from the analysis.

### 3.2.5 Quantification of urinary cytokines

The following human cytokines were quantified in urine samples; interferon gamma (IFN $\gamma$ ), interleukin-1beta (IL-1 $\beta$ ), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10), and tumour necrosis factor alpha (TNF $\alpha$ ). A subset of urine samples were selected to include; 11 control samples, 12 observation samples, as 2 patients at 3 timepoints and 3 patients at 2 timepoints, and 17 Septra samples, as 5 patients at 3 timepoints (baseline, during prophylaxis, and after prophylaxis) and one patient at baseline. Concentrations of cytokines were measured using multiplexed immunoassay kits according to the manufacturer's instructions (EMD Millipore, Burlington, MA, USA). Prior to addition to the kit, urine samples were centrifuged at  $5,000 \times g$  for 5 minutes at room temperature; the supernatant was mixed 1:1 with assay buffer and incubated for 20 minutes at room temperature to neutralize sample and reduce background. Fluorescence was measured on a Bio-Plex 200 system (Bio-Rad, Hercules, CA, USA) using Luminex® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX, USA). Cytokine concentrations (pg/mL) were calculated from standard curves using Bio-Plex Manager v4.1.1 (Bio-Rad).

### 3.2.6 Statistical analysis

All patient demographic and history, urinalysis and bacterial culture, as well as cytokine data, were analysed using GraphPad Prism v7.0a for Mac OS X (GraphPad Software, San Diego, CA, USA). To test for significance in the RUTI group, patient demographic and history data was analysed using one-way analysis of variance (ANOVA) followed by post hoc Tukey's multiple comparisons tests and unpaired t-tests. Significance of

cytokine data was determined using unpaired t-tests to compare control and RUTI participants and repeated measures ANOVA for RUTI time-course data. Pearson's and Spearman's correlation coefficients were used to test for correlation within the dataset.

### 3.3 Results

#### 3.3.1 Patient recruitment and medical history

Participant recruitment began in October 2012 and ended August 2014 for RUTI patients; follow-up continued until August 2015. Recruitment of healthy controls was completed in March 2016. A total of 39 patients were approached about study participation, 37 consented and two declined. In contrast, patient retainment rates were not high; we collected 3-month samples from 21 participants (57%), 6-month from 18 (49%), 9-month from 16 (43%), and 12-month from 14 (38%).

All RUTI patients recruited to the study were females between the ages of 3 and 12 ( $6.8 \pm 2.6$ ). Participants reported experiencing between 3 and 70 ( $11.3 \pm 12.4$ ) symptomatic UTIs over a period of 6 months to 10 years ( $2.8 \pm 2.4$ ) prior to study enrollment. Participants were split into groups for longitudinal analysis based on antibiotic therapy status; antibiotic prophylaxis as Septra ( $n = 18$ ) or nitrofurantoin ( $n = 7$ ), or those that were not prescribed antibiotic prophylaxis (observation,  $n = 12$ ). All control patients were female.

Differences in patient-reported history of UTI and voiding habits were generally not significant between the three groups (Table 3-2); however, patients receiving nitrofurantoin reported more symptomatic UTIs prior to study enrollment than observation patients ( $P = 0.03$ ), voided significantly more often per 24-hour period than patients receiving Septra prophylaxis ( $P = 0.03$ ) and were significantly less likely to report symptoms of abdominal pain associated with UTI than those receiving Septra ( $P = 0.02$ ). Further, patients receiving antibiotics, Septra or nitrofurantoin, reported significantly more UTIs prior to study enrollment than those in the observation group ( $P = 0.01$ ). Almost half of participants (46%) reported constipation.



In regards to the DVSS, the reported incidence of nocturnal enuresis was significantly worse in the nitrofurantoin group relative to both the Septra ( $P = 0.0003$ ) and observation groups ( $P = 0.0006$ ). Urgency was also significantly more common in the nitrofurantoin ( $P = 0.04$ ) and antibiotic prophylaxis (Septra and nitrofurantoin;  $P = 0.02$ ) groups relative to observation. Finally, the total DVSS score was significantly higher in the antibiotic prophylaxis group (Septra and nitrofurantoin) compared to observation ( $P = 0.03$ ).

**Table 3-2. Recurrent UTI patient demographics.**

	Antibiotic Status		
	Observation (n = 12)	Antibiotic Prophylaxis*	
		Septra (n = 18)	Nitrofurantoin (n = 7)
Age	6.8 (± 2.6)	6.9 (± 2.5)	6.3 (± 2.9)
<b>UTI history</b>			
Symptomatic years	1.9 (± 1.6)	3.0 (± 2.3)	3.9 (± 3.2)
No. experienced	5.8 (± 3.2) <sup>†</sup>	11.4 (± 7.9)	20.7 (± 23.8) <sup>‡</sup>
No. febrile	1.8 (± 1.9)	2.3 (± 5.1)	2 (± 3.0)
<b>Reported UTI symptoms</b>			
Dysuria	10 (83%)	13 (72%)	5 (71%)
Fevers	10 (83%)	14 (78%)	5 (71%)
Flank pain	1 (8%)	3 (17%)	0
Abdominal pain	3 (25%)	10 (56%)	0 <sup>§</sup>
<b>Previous UTI Culture<sup>§</sup></b>			
<i>E. coli</i>	9	11	6
<i>E. faecalis</i>	1	3	3
<i>Proteus mirabilis</i>	1	4	0
<i>Klebsiella</i> sp.	0	1	1
<i>Staphylococcus</i> sp.	0	1	0
<b>Voiding habits</b>			
24 hr void frequency	6 (± 2.3)	5.8 (± 2.4)	8.9 (± 3.4) <sup>  </sup>
Nocturnal enuresis	5 (42%)	10 (56%)	4 (57%)
Diurnal enuresis	3 (25%)	7 (39%)	3 (43%)
OAB Signs	5 (42%)	9 (50%)	5 (71%)
Straining to void	1 (8%)	5 (28%)	0
Constipation	4 (33%)	10 (56%)	3 (43%)
PEG3350 prescribed	3 (25%)	10 (56%)	4 (57%)

\*Prescribed as nitrofurantoin at 2 mg/kg and Septra at a trimethoprim dose of 2 mg/kg.

<sup>†</sup>P < 0.05 in comparison to antibiotic prophylaxis (Septra and nitrofurantoin) by unpaired t-test with Welch's correction.

<sup>‡</sup>P < 0.05 in comparison to observation by one-way ANOVA with Tukey's multiple comparisons test.

<sup>§</sup>Previous culture results were obtained through the consultation request and are not available for all patients, thus no percentage is represented.

<sup>||</sup>P < 0.05 in comparison to Septra by one-way ANOVA with Tukey's multiple comparisons test.

Abbreviations: hr, hours; No., number; OAB, overactive bladder; PEG3350, polyethylene glycol 3350.

### 3.3.2 Urinalysis

All samples were negative for glucose, and urine from control participant 6 (C6) was the only to test positive for ketones. At baseline, specific gravity was significantly higher in healthy controls compared to recurrent UTI patients as both a general group ( $P = 0.0001$ ) and specifically those receiving Septra ( $P = 0.004$ ).

### 3.3.3 Bacterial culture

Total viable plate counts were not significantly different for FV or MS urine on CHROMagar Orientation nor were counts from FV urine on CBA following 48-hour incubation. However, total viable counts from the MS urine of children with RUTI were significantly higher on CBA in comparison to the urine of healthy children ( $P = 0.04$ ).

Presumptive identification was completed using CHROMagar Orientation, a chromogenic culture medium optimised for the enumeration and differentiation of uropathogens (Tables 3-3 and 3-4). Based on these presumptive identifications, we identified *Citrobacter* in urine samples from patients 8, 12, and C17 at baseline, always below the limit of quantitation (LOQ). Further, we only isolated *Proteus mirabilis* from one patient following Septra antibiotic prophylaxis. *E. coli* and *E. faecalis* were the two most commonly isolated uropathogens. Notably, 19% of MS urine samples met the diagnostic cutoff for UTI (Table 3-3; Figures 3-1 and 3-2;),  $10^5$  bacteria of one species, yet patients remained symptom-free suggesting this was a state of ABU.

*E. faecalis* viable counts were not significantly different between RUTI and control urines, yet enterococci were significantly more likely to be isolated in RUTI samples ( $P = 0.04$ ; Figures 3-1 and 3-3). In comparison, *E. coli* viable counts were significantly higher in RUTI relative to control urine ( $P < 0.0001$ ), yet there was no difference in incidence. Further, there was a positive correlation between the CFU of *E. faecalis* and non-*E. coli* uropathogens in both healthy and RUTI urines (Figure 3-5), the correlation coefficient was 0.52 from FV urine ( $P = 0.0003$ , 95% CI 0.27-0.71) and 0.4 from MS urine ( $P = 0.01$ , 95% CI 0.10-0.63).

Over the course of the study, *E. faecalis* was detected in the FV urine of 22 RUTI patients (n = 35, 63%) and in the MS urine of 15 (n = 33, 45%; Tables 3-3 and 3-4). Additionally, antibiotic prophylaxis mediated a slight decrease in uropathogen load; however, bacterial counts returned to pre-antibiotic levels following cessation of therapy (Figures 3-2 and 3-4). The majority of cultures were polymicrobial, and multiple colony morphologies were noted; only uropathogens were presumptively identified.

Table 3-3. Viable bacterial counts from MS urine.

Samples with uropathogen growth (%)										
Visit 1*			Visit 2*			Visit 3*			Visit 4*	
Detected	Above threshold†	n	Detected	Above threshold†	n	Detected	Above threshold†	n	Detected	Above threshold†
<b>HEALTHY</b>										
<i>Enterococcus</i>	15.8			5.3						
<i>E. coli</i>	26.3			0						
<i>K/E</i>	15.8	0	19							
<i>S. saprophyticus</i>	31.6			5.3						
<i>P/S</i>	73.7	0								
<b>RUTI</b>										
<b>Observation</b>										
<i>Enterococcus</i>	40	0	75	25	100	100				
<i>E. coli</i>	20	0	25	0	0	0				
<i>K/E</i>	30	10	0	0	4	0	0	1		
<i>S. saprophyticus</i>	20	0	25	0	0	0				
<i>P/S</i>	70	0	50	0	0	0				
<b>Antibiotic prophylaxis</b>										
<i>Enterococcus</i>	47.1	11.8	42.9	14.3	20	20	0	27.3	0	
<i>E. coli</i>	29.4	5.9	42.9	14.3	53.3	26.7	63.6	54.5		
<i>K/E</i>	17.6	0	17	0	7	26.7	6.7	15	0	11
<i>S. saprophyticus</i>	5.9	0	0	0	20	6.7	9.1	0		
<i>P/S</i>	70.6	0	57.1	0	46.7	27.3	0			

\*The timeline for patients receiving antibiotic prophylaxis is adjusted as follows; visit 1, prior to antibiotic prophylaxis; visit 2, patient on antibiotics; visits 3 and 4, patient is no longer receiving prophylaxis.

†Urine samples with bacterial counts of one species at or above the clinical threshold, defined as >100,000 of that single species.  
Abbreviations: *K/E*, *Klebsiella/Enterobacter*; *P/S*, *Pseudomonas/S. aureus*.

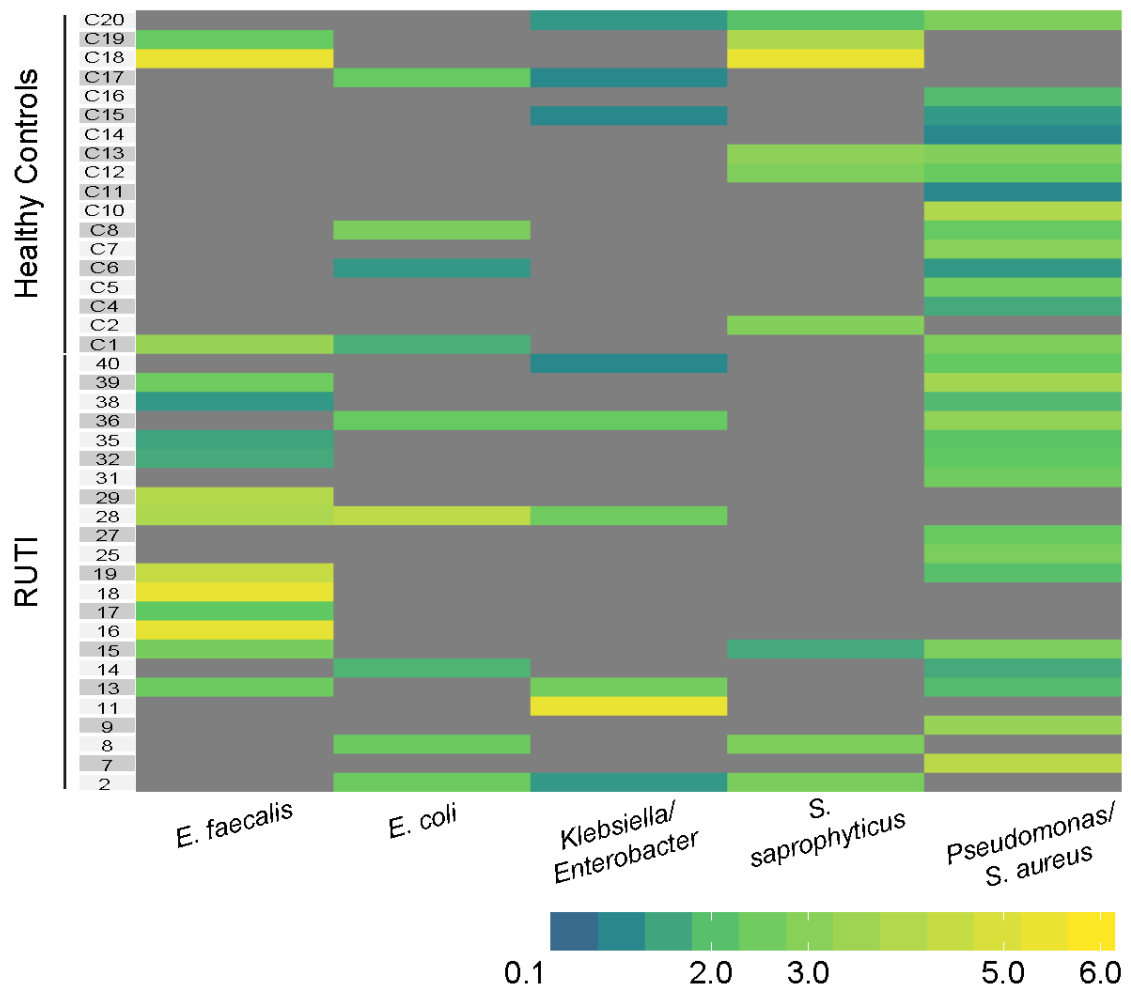
Table 3-4. Viable bacterial counts from FV urine.

Urine samples with uropathogen growth (%)										
Visit 1*			Visit 2*			Visit 3*			Visit 4*	
Detected	Above threshold <sup>†</sup>	n	Detected	Above threshold <sup>†</sup>	n	Detected	Above threshold <sup>†</sup>	n	Detected	Above threshold <sup>†</sup>
<b>HEALTHY</b>										
<i>Enterococcus</i>	23.5			11.8						
<i>E. coli</i>	47.1			0						
<i>K/E</i>	0	17								
<i>S. saprophyticus</i>	29.4			5.9						
<i>P/S</i>	70.6			0						
<b>RUTI</b>										
<b>Observation</b>										
<i>Enterococcus</i>	60	0	60	20	20	40	20	0	0	0
<i>E. coli</i>	50	10	80	0	0	40	0	0	0	0
<i>K/E</i>	10	10	20	0	5	0	0	5	0	1
<i>S. saprophyticus</i>	30	0	20	0	0	20	0	0	0	0
<i>P/S</i>	70	0	80	0	0	0	0	100	0	0
<b>Antibiotic prophylaxis</b>										
<i>Enterococcus</i>	58.8	5.9	50	0	0	35.3	0	25	0	0
<i>E. coli</i>	41.2	0	60	10	10	64.7	29.4	75	75	75
<i>K/E</i>	23.5	0	20	0	10	11.8	0	8.3	0	12
<i>S. saprophyticus</i>	5.9	0	0	0	0	17.6	5.9	0	0	0
<i>P/S</i>	58.8	0	80	0	0	52.9	5.9	41.7	0	0

\*The timeline of samples from patients receiving antibiotic prophylaxis is adjusted as follows; visit 1, prior to antibiotic prophylaxis; visit 2, patient on antibiotics; visits 3 and 4, patient is no longer receiving prophylaxis.

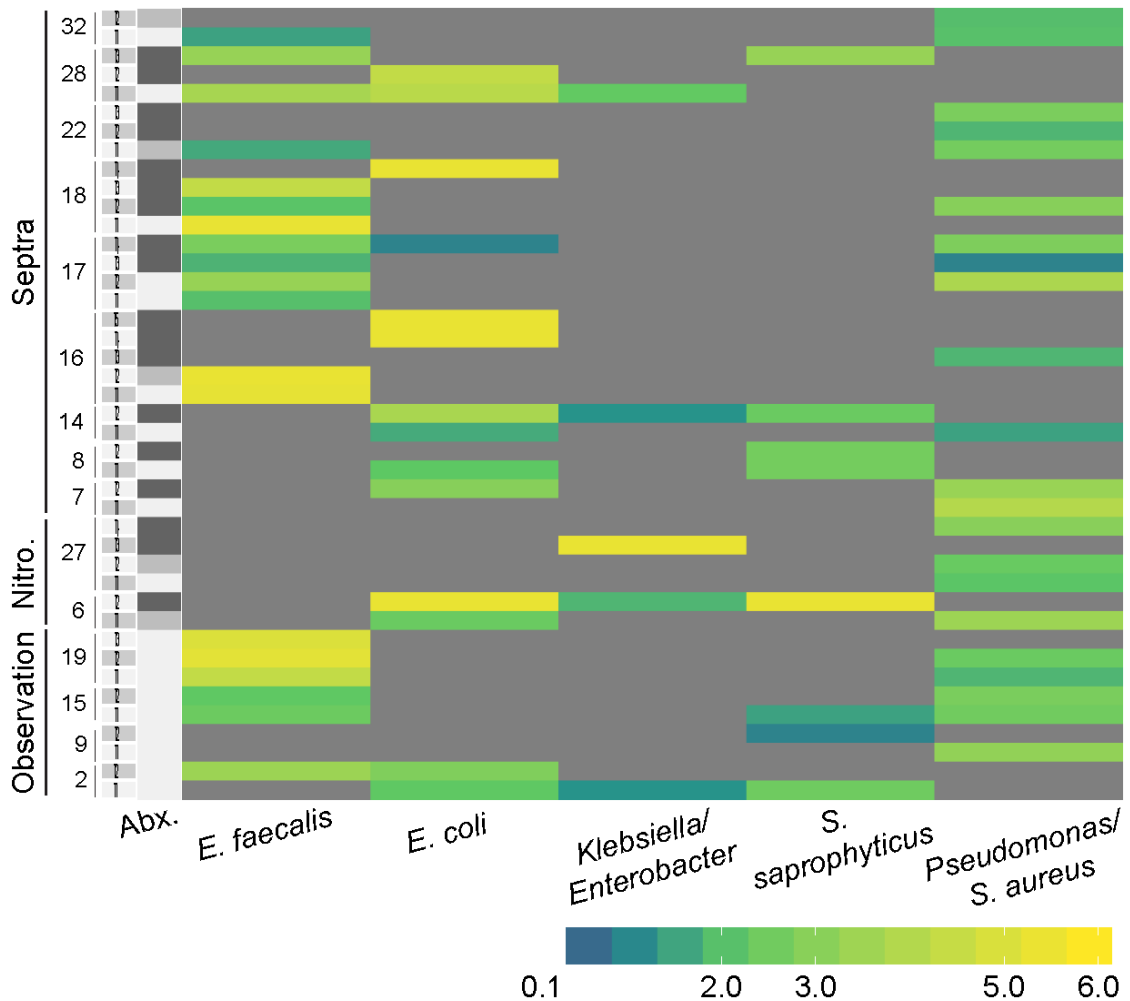
<sup>†</sup>Urine samples with bacterial counts of one species at or above the clinical threshold, defined as >100,000 of that single species.  
Abbreviations: *K/E*, *Klebsiella/Enterobacter*; *P/S*, *Pseudomonas/S. aureus*.





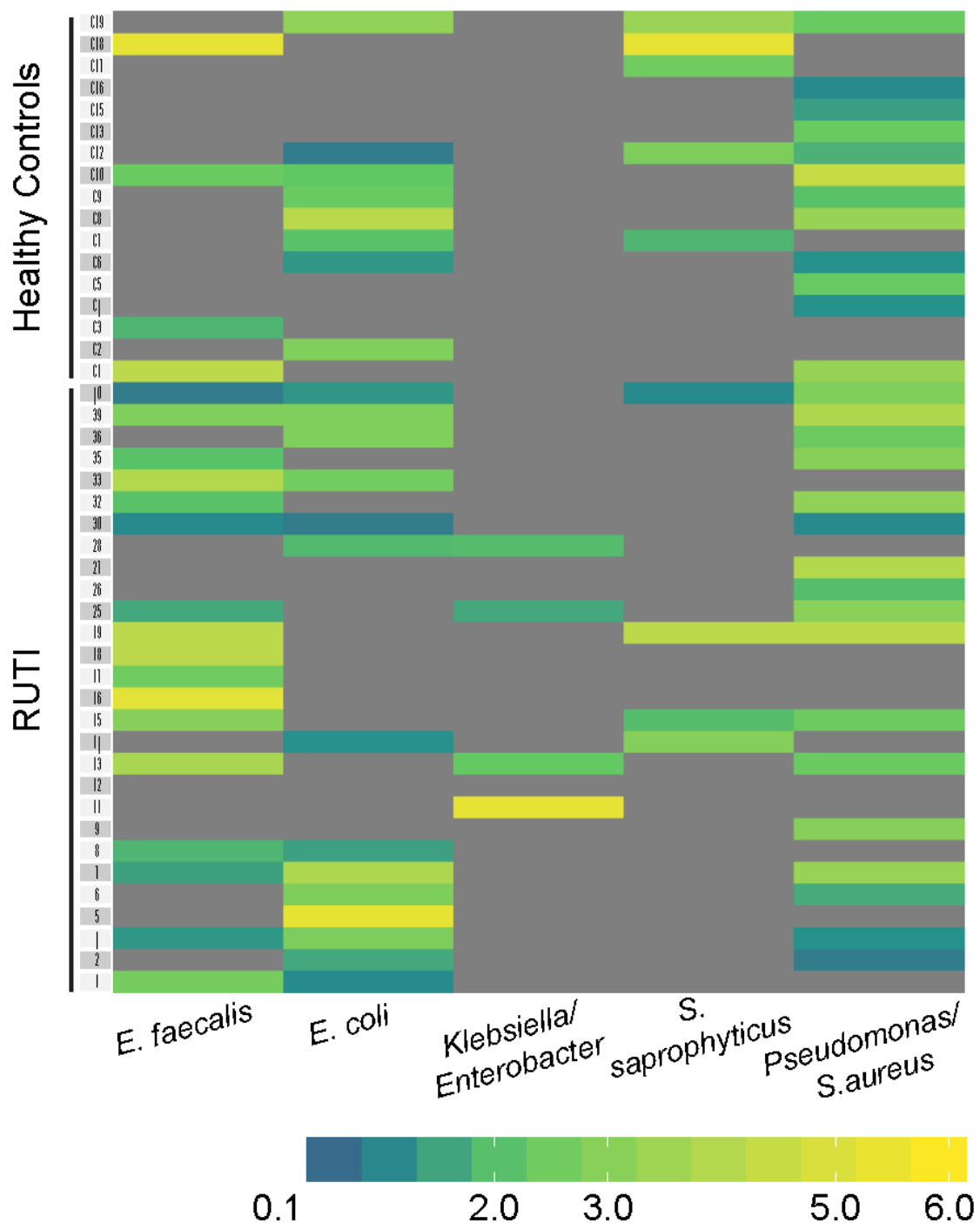
**Figure 3-1. Uropathogen isolation from midstream urine of healthy and RUTI patients.**

The enumeration and presumptive identification of *E. faecalis*, *E. coli*, *Klebsiella/Enterobacter*, *Staphylococcus saprophyticus*, and *Pseudomonas/S. aureus* was completed with CHROMagar orientation from the midstream urine of healthy controls and patients with a history of RUTI at baseline. Data is presented as log<sub>10</sub> CFU/mL; gray boxes denote species that were not detected in that sample.



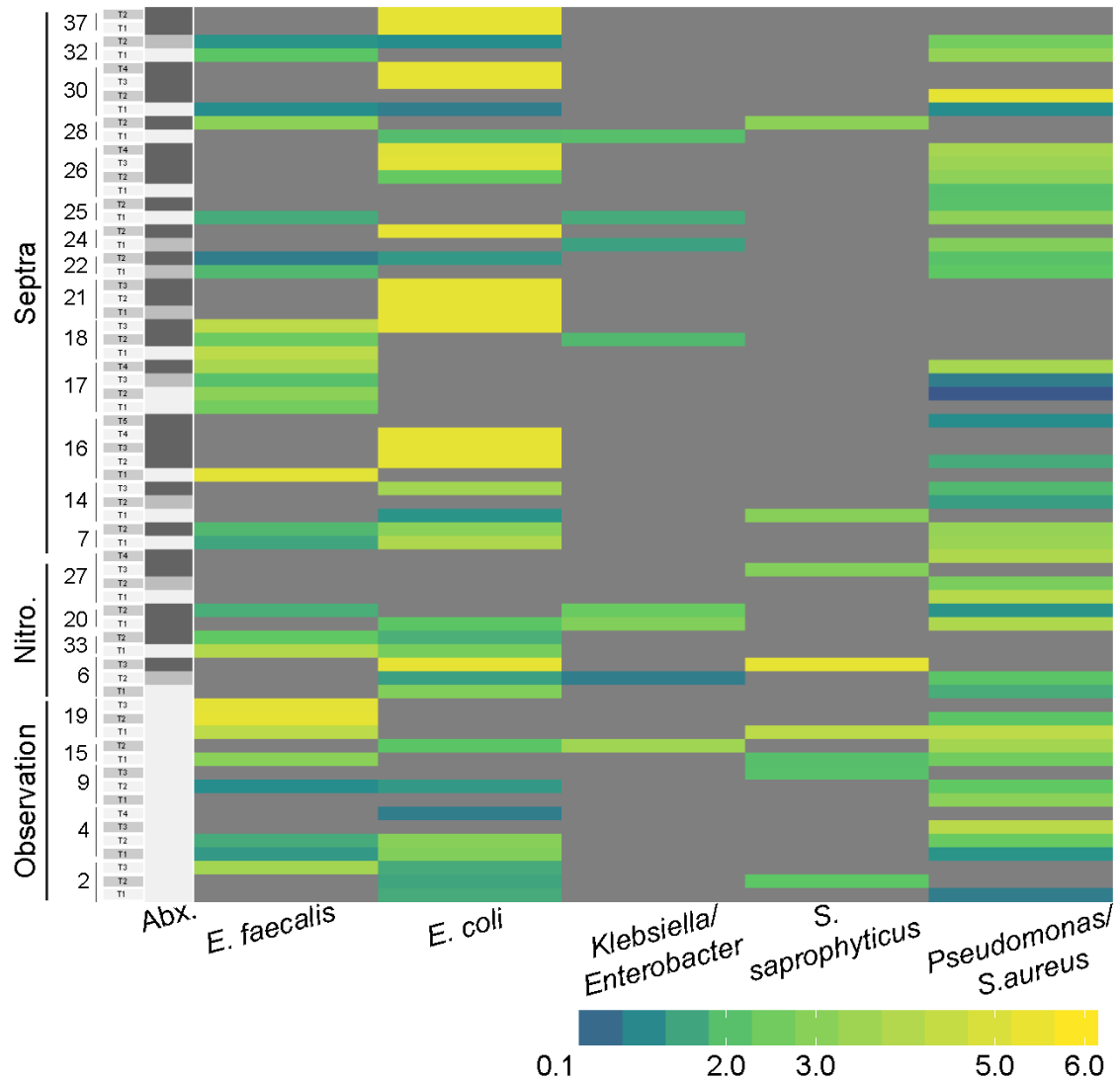
**Figure 3-2. Uropathogen isolation from midstream urine of RUTI patients over time.**

The enumeration and presumptive identification of *E. faecalis*, *E. coli*, *Klebsiella/Enterobacter*, *S. saprophyticus*, and *Pseudomonas/S. aureus* was completed with CHROMagar orientation from the midstream urine of patients with a history of RUTI over a 12-month period. Patients were observed clinically (Observation), or received nitrofurantoin (Nitro.) or TMP/SMX (Septra) prophylaxis. Antibiotic status is indicated to the left of the heatmap (Abx.); no antibiotic (light gray), on at antibiotic at sample collection (medium gray), or off antibiotic (dark gray). Data is presented as  $\log_{10}$  CFU/mL; gray boxes denote species that were not detected in that sample.

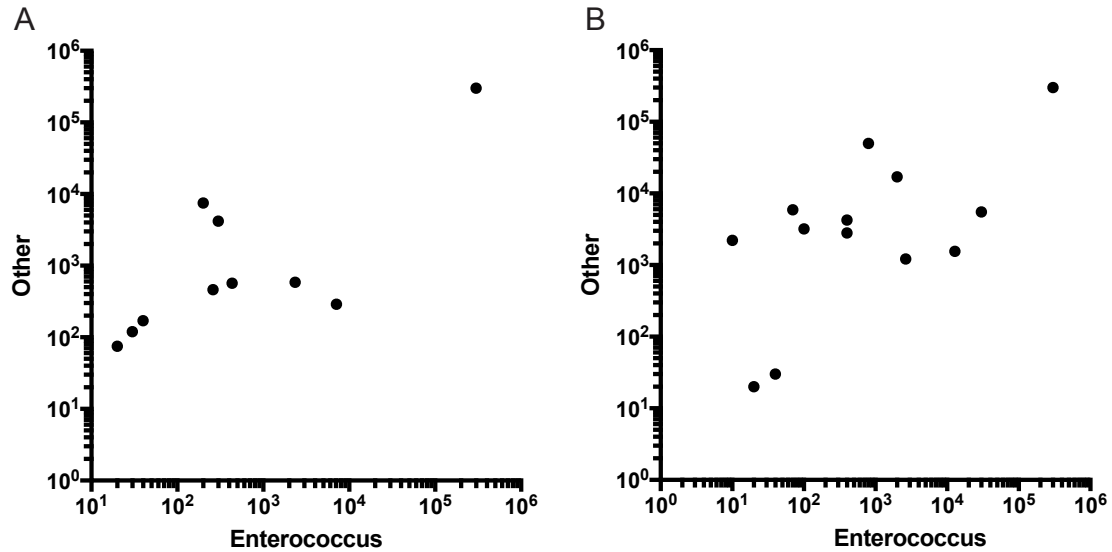


**Figure 3-3. Uropathogen isolation from first-void urine of healthy and RUTI patients.**

The enumeration and presumptive identification of *E. faecalis*, *E. coli*, *Klebsiella/Enterobacter*, *Staphylococcus saprophyticus*, and *Pseudomonas/S. aureus* was completed with CHROMagar orientation from the first-void urine of healthy controls and patients with a history of RUTI at baseline. Data is presented as log<sub>10</sub> CFU/mL; gray boxes denote species that were not detected in that sample.



**Figure 3-4. Uropathogen isolation from first-void urine of RUTI patients over time.** The enumeration and presumptive identification of *E. faecalis*, *E. coli*, *Klebsiella/Enterobacter*, *S. saprophyticus*, and *Pseudomonas/S. aureus* was completed with CHROMagar orientation from the first-void urine of patients with a history of RUTI over a 12-month period. Patients were observed clinically (Observation), or received nitrofurantoin (Nitro.) or TMP/SMX (Septra) prophylaxis. Antibiotic status is indicated to the left of the heatmap (Abx.); no antibiotic (light gray), on at antibiotic at sample collection (medium gray), or off antibiotic (dark gray). Data is presented as log<sub>10</sub> CFU/mL; gray boxes denote species that were not detected in that sample.



**Figure 3-5. Correlation between *Enterococcus* and non-*E. coli* bacterial load in urine.**

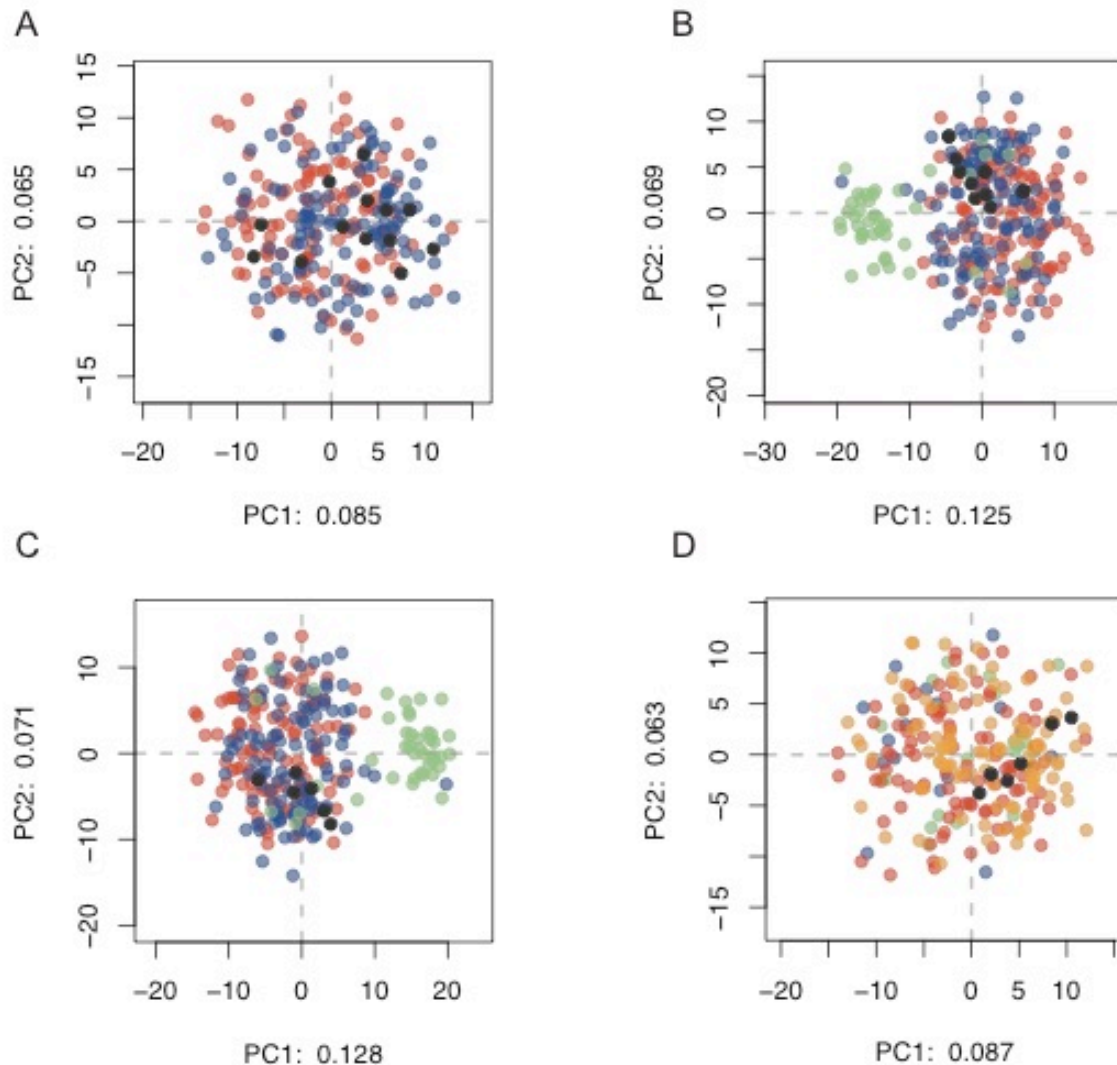
Enumeration and presumptive identification of uropathogens from urine was completed using CHROMagar orientation. Of the uropathogens identified, the bacterial load of *Enterococcus* and the non-*E. coli* bacteria were positively correlated in both midstream (A;  $r = 0.40$ ,  $P = 0.01$ ) and first-void (B;  $r = 0.52$ ,  $P = 0.0003$ ) urine. Data is presented as CFU/mL. Correlation was determined using Pearson's correlation coefficient.

### 3.3.4 16S rRNA sequencing

Our initial data exploration indicated there were no differences in the microbial communities of FV or MS urine from any of the study participants. Additionally, the DNA extraction and no template controls grouped with the urine samples in our principle component analysis (PCA) plot (Figure 3-6, A). To overcome this, we attempted to remove amplicon sequence variants (ASVs) that were of significantly higher relative abundance in the experimental blanks compared to urine samples; however, this did not improve data separation.

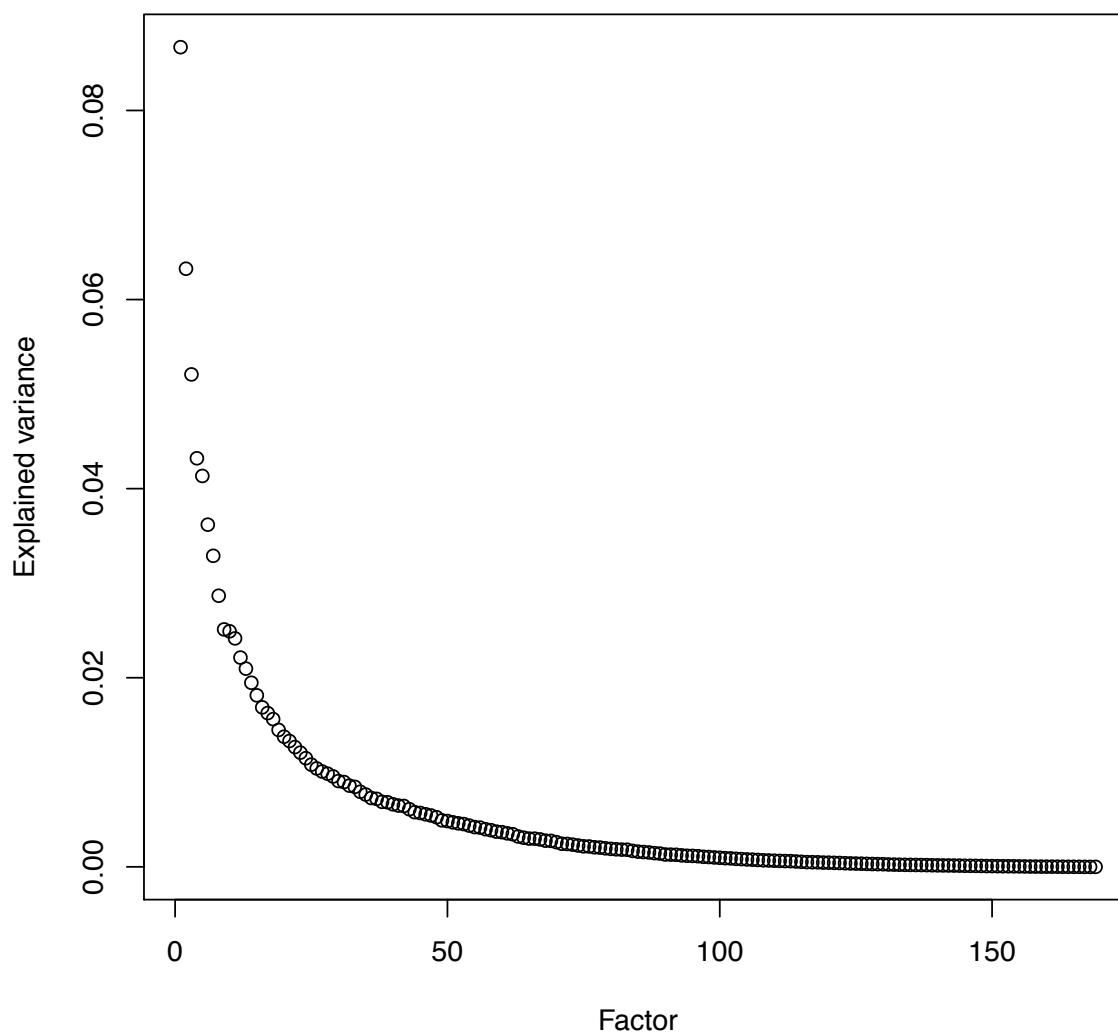
Given that the urine samples were processed alongside chemostat samples, we combined the individual datasets to assess for differences between urine, blanks, and chemostat samples. The resulting PCA plot demonstrated a separation between urine and chemostat samples along principle component 1 (PC1); however, PC1 accounted for only 12.5% of the variance (Figure 3-6, B). We were able to determine that over 90% of the variance was attributed to a single ASV, which corresponded to *Escherichia/Shigella* (data not shown). This ASV was removed, and the dataset reanalysed.

The resulting PCA (Figure 3-6, C) continued to demonstrate grouping of the experimental blanks with the urine samples, and the separation of urine and chemostat samples along PC1 could be explained by less than 13% of the variance. Given that the PCA takes into account all the samples analysed, we removed the chemostat samples and repeated the CoDaSeq analysis and PCA plot (Figure 3-6, D). Similar to the initial PCA, no separation was noted between FV or MS urine, regardless of disease status, and experimental blanks continued to group with the samples. A subsequent scree plot of the data indicated that no single factor accounts for the lack of variance (Figure 3-7).



**Figure 3-6. Principle component analyses of 16S rRNA gene sequencing data.**

Variance in the 16S rRNA gene sequencing data was visualised as a series of principle component analyses (PCA). **A** | Experimental blanks (black) did not group separately from the first-void (FV; red) or midstream urine (MS; blue). **B** | Urinary and chemostat datasets were combined to assess for contamination. Most chemostat samples (green) grouped separately of the urine and blanks (FV, red; MS, blue; blanks, black) along principle component 1 (PC1; 0.125). **C** | Amplicon sequencing variant 1 (ASV1) was removed from the urine-chemostat dataset; most chemostat samples continued to group separately from the urine and blanks along PC1 (0.128). **D** | Variance of urine samples and blanks was visualised following the removal of ASV1 from the data set. Healthy FV (blue), healthy MS (green), recurrent urinary tract infection (RUTI) FV (red), RUTI MS (orange), and experimental blank (black) samples were inseparable by PCA.



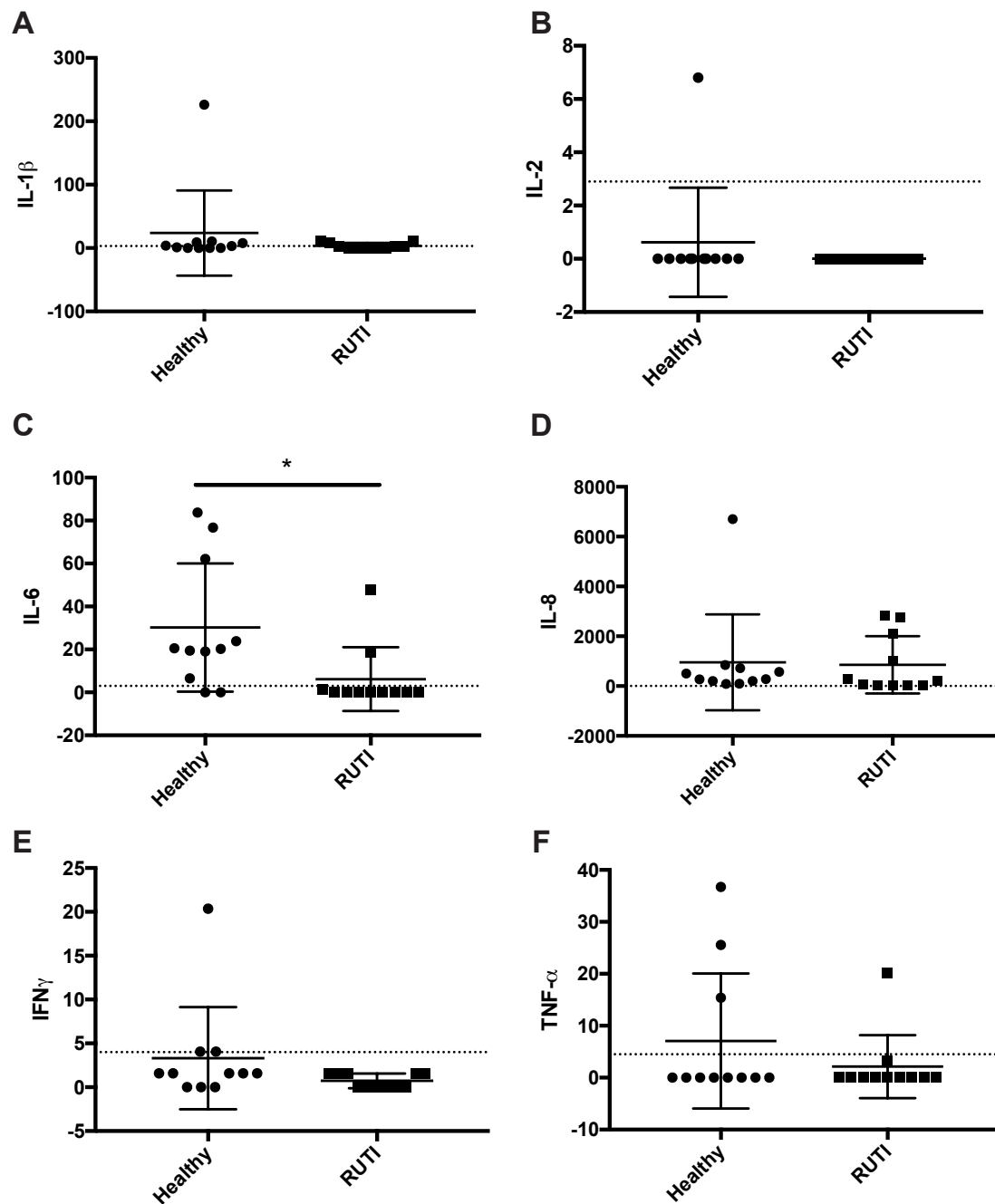
**Figure 3-7. Scree plot of 16S rRNA gene sequencing data from urine and experimental blanks.**

A plot of the fraction of total variance associated with each principle component (PC) is presented for the urinary dataset following the removal of amplicon sequencing variant 1 (ASV1). The y-axis of the graph demonstrates that no single PC is associated with the datasets variation.



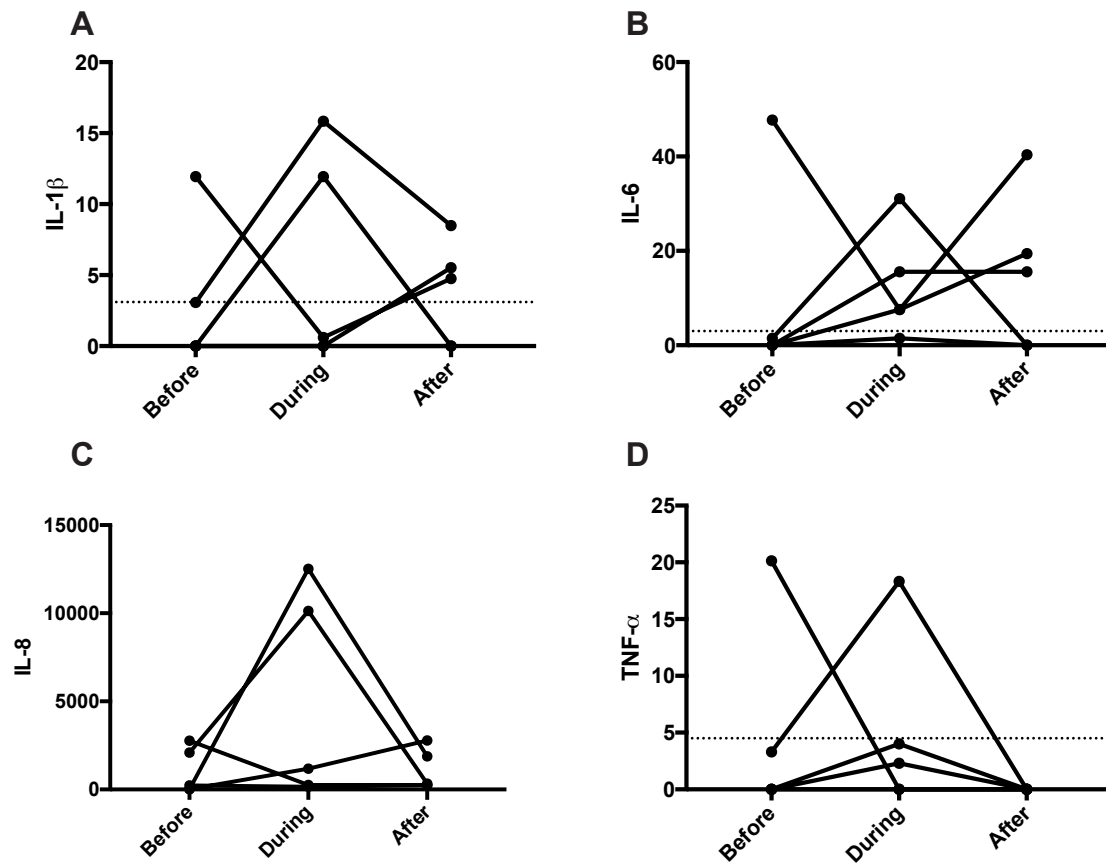
### 3.3.5 Urinary cytokines

Cytokine levels were below the LOQ in most of the samples assayed (Figure 3-8, 3-9, and 3-10). There were no significant differences in the concentration of IL-1 $\beta$ , IL-2, IL-8, IL-10, IFN $\gamma$ , or TNF $\alpha$  (Figure 3-8 and 3-9); however, IL-6 was significantly higher in the healthy group compared to RUTI at baseline ( $P = 0.03$ ). Control 8 (C8), was the only sample we were able to determine the concentration of IL-2 in (6.8  $\mu\text{g/mL}$ ), and had the highest concentration of IL-1 $\beta$  (226.0  $\mu\text{g/mL}$ ), IFN $\gamma$  (20.35  $\mu\text{g/mL}$ ), and IL-10 (17.38  $\mu\text{g/mL}$ ). There was also no significant difference in cytokine concentrations across the longitudinal samples (Figure 3-8 and 3-10, B).



**Figure 3-8. Baseline urinary pro-inflammatory cytokines.**

Cytokine concentrations are presented in pg/mL from the urine of healthy and RUTI participants at baseline. The dashed line represents the lower limit of quantitation, and corresponds to 3.1 for IL-1 $\beta$  (A), 2.9 for IL-2 (B), 3.0 for IL-6 (C), 3.6 for IL-8 (D), 4.0 for IFN $\gamma$  (E), and 4.5 pg/mL for TNF $\alpha$  (F). Urinary cytokine concentrations were determined for each patient in duplicate using Luminex® xMAP fluorescent bead-based technology. Significance was determined using unpaired t-tests (\*, P < 0.05).



**Figure 3-9. Urinary pro-inflammatory cytokines over the course of Septra prophylaxis.**

Urinary cytokine concentrations of five patients over the course of Septra prophylaxis are presented in pg/mL. Samples were collected at baseline (before), 3-months into prophylaxis (during), and during the antibiotic wash-out period (after). The dashed line represents the lower limit of quantitation, and corresponds to 3.1 for IL-1 $\beta$  (A), 3.0 for IL-6 (B), 3.6 for IL-8 (C), and 4.5 pg/mL for TNF $\alpha$  (F). Urinary cytokine concentrations were determined for each patient in duplicate using Luminex® xMAP fluorescent bead-based technology. Significance was determined using repeated-measures one-way ANOVA.



### 3.4 Discussion

In this pilot study, we followed paediatric patients with RUTI over the course of 12-months, during which patients either received antibiotic prophylaxis as TMP/SMX or nitrofurantoin, or were clinically observed. We demonstrated the presence of uropathogens, including *E. coli* and *E. faecalis*, in the urine of both healthy and RUTI participants. Additionally, we demonstrated prophylaxis may not be having its desired effect, as bacterial loads increase following cessation of therapy and have no measurable effect on urinary cytokine levels.

Participant recruitment began in October 2012 and ended August 2014 for RUTI patients; follow-up continued until August 2015. Recruitment of healthy controls was completed in March 2016. We had initially projected a 6-month recruitment period; however, it was almost two years before we successfully recruited 37 patients. This was a direct reflection of the available population, as we had a recruitment rate of over 95%. In total, 39 patients were approached and 37 agreed to participate, those who declined cited costs associated with the increased frequency of clinic visits and a lack of interest.

In contrast, our participant retainment rates were quite low, dropping from 56% at 3-months to below 40% by the 12-month mark. One participant officially dropped out of the study after three months, while the rest ceased to attend appointments. In comparison, the PRIVENT, RIVUR, and IRIS prophylaxis trials lost less than 8% of patients to follow-up<sup>10,33,54</sup>. As our study was designed as a pilot and was initially unfunded, we were unable to offer incentives; participants and their families were responsible for all travel and parking costs, and prescriptions were at personal cost or covered by family drug plans. Procedures, such as uroflowmetry, ultrasound, and/or VCUG were within the standard of care and thus covered by the Ontario Health Insurance Plan (OHIP). In an effort to increase retainment, administrative staff reached out to participants prior to appointments to provide a reminder. Further, all study associated appointments were scheduled at recruitment and the family mailed a letter with the prospective dates (Appendix C). While the loss of patients to follow-up was detrimental to the study outcome, it was not out of character for this clinic, which regularly sees 10-20% of patients fail to attend appointments.

Given that the decision to begin antibiotic prophylaxis or clinical observation was based on the clinical experience of the attending urologist, we expected significant differences in patient histories between the observation and prophylaxis groups. Unsurprisingly, patients in the prophylaxis group reported significantly more UTIs prior to study enrollment than those in the observation group (Table 3-2). This is likely an indicator of perceived RUTI severity; however, there were no significant differences in the number of febrile UTIs or UTI-associated symptoms.

Similarly, patients receiving nitrofurantoin reported more severe nocturnal enuresis and urgency in the DVSS. The total DVSS score was also significantly higher in the prophylaxis group relative to observation; this was expected given clinician bias in the use of antibiotic prophylaxis when BBD is a suspected contributor to RUTI. Indeed, BBD is a well-characterised risk factor for the development of RUTI in children<sup>4</sup>; however, to the best of our knowledge, no research has linked BBD to success rates in antibiotic prophylaxis.

In contrast, the relationship between UTI-associated abdominal pain and choice of prophylactic agent was unexpected, as was the relationship between voiding frequency and prophylactic choice. Neither of these participant characteristics were significantly different between the observation and prophylaxis groups; thus, this observation is not likely to be clinically relevant. Further, clinical guidelines recommend Septra as the first choice for antibiotic prophylaxis, unless local antibiograms indicate TMP/SMX resistance is high in uropathogens or previous urine culture results have isolated TMP/SMX resistant strains<sup>13</sup>.

In regards to the urinalysis results, all samples were negative for glucose, indicating none of our patients had mismanaged or undiagnosed diabetes, which is a risk factor for RUTI given the increased nutrient availability. Specific gravity was significantly lower, yet still within the normal range, in RUTI patients relative to healthy controls; however, this is likely a reflection of the collection conditions. Patients with a history of RUTI provided urine in the clinic, thus urinalysis tests were conducted on the sample immediately, while healthy patients often collected the urine at home and brought the sample to the study

site. The time between urine collection and urinalysis was significantly longer for healthy urine samples, which is associated with loss of test accuracy<sup>55,56</sup>. Alternatively, specific gravity is generally considered a measure of hydration<sup>57</sup>, RUTI patients were asked to arrive at the clinic with a full bladder, it is likely that parents increased fluid intake prior to the appointment for this purpose.

Bacterial enumeration and presumptive identification was completed with CHROMagar Orientation, a chromogenic culture medium optimized for uropathogens. Notably, *E. faecalis* grows as teal colonies, *E. coli* as dark pink, *Staphylococcus saprophyticus* as light pink, *Klebsiella/Enterobacter* as metallic blue, *Citrobacter* as dark blue with a red halo, *Proteus mirabilis* is notable for its brown halo, and both *Staphylococcus aureus* and *Pseudomonas* grow as white/cream colonies on these agar plates. Thus, enumeration and presumptive identification of these common uropathogens can be made within 24 hours of urine collection. CHROMagar Orientation has been extensively validated and is used by many diagnostic laboratories<sup>58-60</sup>. Using this strategy, *E. coli* and *E. faecalis* were the most commonly isolated uropathogens from our samples (Figure 3-1, 3-2, 3-3, and 3-4), which corresponds with *E. coli* and *E. faecalis* being the most commonly reported agents of pre-study culture-proven UTI (Table 3-2). Notably, 19% of MS urine samples met the diagnostic cutoff for UTI,  $>10^5$  CFU/mL of one bacterial species, yet patients remained symptom free, suggesting this was a state of ABU.

Asymptomatic bacteriuria is generally considered a protective state and eradication of the ABU-associated bacteria, particularly enterococci, is associated with increased risk of symptomatic UTI<sup>15,61-64</sup>. Our culture results suggest that antibiotic prophylaxis does not completely eradicate uropathogens, and that cessation of prophylaxis may be associated with an increase in bacterial load (Figures 3-2 and 3-4). These results support research indicating ABU should not be treated<sup>15,61-64</sup>, and implies the diagnostic threshold for ABU, in patients with a history of RUTI, may be too high.

Interestingly, total viable counts were not significantly different between the healthy and RUTI group at baseline on CHROMagar Orientation for either FV or MS urine; yet counts from MS urine on CBA plates were significantly higher in the RUTI population.

Likewise, *E. faecalis* counts were not significantly different between RUTI and control urines; however, *E. faecalis* was more likely to be isolated from RUTI urine. In comparison, *E. coli* CFUs were significantly higher in RUTI urine, yet there was no difference in incidence. These findings raise questions in regards to what constitutes a “healthy” urinary microbiota in the paediatric population.

Study of the urinary microbiota in adult women has described *E. faecalis* in the urine of patients with interstitial cystitis/bladder pain syndrome (IC/BPS), neurogenic bladder, and in the majority of non-symptomatic patients with a history of UTI, yet none have identified *E. faecalis* in the urine of healthy women<sup>15,65-68</sup>. Thus, *E. faecalis* presence in the urinary tract may be an indicator of ill-health. A clinical study of the urinary microbiota in renal transplant patients found *E. faecalis* to be significantly abundant in the urine of patients prior to development of symptomatic UTI<sup>28</sup>.

The data also indicates a positive correlation between the bacterial load of *E. faecalis* and non-*E. coli* uropathogens (Figure 3-5), which is notable given the prevalence of enterococci in polymicrobial UTI<sup>69</sup>. Of note, *Enterococcus* sp. were found in low abundance within almost one-third of *E. coli*-dominated acute UTI samples<sup>24</sup>, suggesting enterococci may be a significant contributor to both mono- and polymicrobial UTI. Indeed, the majority of our urine cultures were polymicrobial, with multiple colony morphologies noted per urine sample; however, only uropathogens were presumptively identified. We documented a variety of pinpoint colonies on our plates (CHROMagar and CBA), yet were unable to subculture most of these bacteria. These colonies may represent small colony variants, or, more likely, are dependent on the byproducts of other bacteria for *in vitro* growth as their colony counts were discontinuous with increasing dilutions.

Unfortunately, we were unable to assess the polymicrobial nature of our urine samples using the 16S rRNA gene sequencing data. Our initial exploratory analysis indicated no significant differences in variance between the urine and experimental blanks (Figure 3-6, A), thus we attempted to remove contaminating ASVs; however, this did not improve data separation. Given that DNA extraction, PCR amplification, and ultimately



sequencing were completed in parallel with chemostat samples, we made the assumption that the higher abundance chemostat samples were the source of the contamination. To test this, we combined the individual datasets and completed a second PCA (Figure 3-6, B). The urine and chemostat groups did separate along PC1; however, this only accounted for 12.5% of the variance and we should expect a much larger difference between urine and chemostat samples. We were able to attribute a large portion of this variance to ASV1, which we subsequently removed from the analysis. Notably, when data is analysed as proportions, removal of a single attribute disrupts the proportions of the remaining data; however, as our data was analysed as compositions<sup>49,50,52,53</sup>, we were able to remove ASV1 without interfering with the interpretation of the remaining ASVs.

Following the removal of ASV1, we noted no significant changes in the urine-chemostat dataset (Figure 3-6, C), nor was there separation of the urine and experimental blanks subsequent to removal of the chemostat samples (Figure 3-6, D). Given the low abundance of the urine samples we would have expected the experimental blanks to group separately, thus we concluded the urinary data was contaminated by the higher-abundance chemostat samples and would not convey meaningful results.

Finally, we measured urinary cytokine levels as an indication of bladder inflammation and bacterial interactions with the urothelium. Given that bacterial attachment to urothelial cells is one of the first steps in bacterial colonization, the urothelium acts as the one body's early infection defence systems. *In vitro* experiments have demonstrated the production and release of cytokines by the urothelium in response to whole bacteria and purified bacterial toxins, such as pyocyanin or lipopolysaccharide (LPS)<sup>70-72</sup>. Of interest to our group, *E. faecalis* infection was associated with increased expression of IL-6, IL-12, G-CSF, IL-1 $\beta$ , IL-1 $\alpha$ , and IL-17 in a murine model of catheter-associated UTI<sup>73,74</sup>, and in humans bacterial cystitis correlates with increased urinary IL-1 $\alpha$ , IL-1 $\beta$ , TNF $\alpha$ , IL-6, and IL-8<sup>72,75</sup>. Furthermore, urinary IL-6 and IL-8 have shown prognostic value for paediatric UTI<sup>76</sup>, and been identified as markers of disease severity<sup>75</sup>.

Contradictory to these reports, our data demonstrates significantly higher concentrations of IL-6 in the urine of healthy children. While statistically significant, the biological

relevance of our cytokine results is debatable, as both the n-value and concentration differential were relatively small. There are however, a number of potential explanations for the decrease in urinary IL-6; first of all, RUTI patients may not be able to mount an effective immune response to uropathogens and/or low-level inflammation may be beneficial to the maintenance of homeostasis, which aligns with the genetic aspects of RUTI<sup>14,77</sup>. Alternatively, uropathogens may be interfering with the bladder's immune response through a dampening of cytokine levels and/or interruption of urothelial signaling; this may help explain the increase in cytokine levels associated with symptomatic UTI relative to ABU<sup>75</sup>. Interestingly, most cytokines were either undetected in the urine samples or detected below the LOQ (Figure 3-8, 3-9, and 3-10), regardless of the uropathogen load within the sample. Cytokines levels were also not significantly altered over the course of prophylaxis, indicating antibiotic prophylaxis may not be having its desired effects.

### 3.4.1 Conclusions

Originally designed as a pilot, the goal of this clinical study was to explore the urinary prevalence of enterococci in paediatric patients with RUTI and determine if antibiotic prophylaxis would induce alterations in the microbial communities of this population. Our culture results demonstrated the presence of uropathogens in the urine of healthy children and children with a history of RUTI, although none of our participants were experiencing symptomatic-UTI. Further, antibiotic prophylaxis appears to temporarily decrease urine CFUs, yet following cessation of therapy the bacterial load drastically increases, similar to what is noted with the treatment of ABU. It is possible that these low-levels of bacteria noted in our RUTI samples behave similarly to ABU strains. This warrants future study, especially considering the high rates of prophylaxis-associated recurrent infections. The positive correlation of *E. faecalis* with non-*E. coli* uropathogens is also of interest and highlights the contribution of *E. faecalis* to polymicrobial UTI. Indeed, most polymicrobial urine samples are considered contamination, yet data exists suggesting enterococci contribute to the pathogenesis of other bacteria and symptom onset may be a result of a disruption in homeostasis<sup>28</sup>. Finally, the decreased urinary IL-6 noted in our RUTI population may be indicative of bacterial interference with the bladder

immune response. Given the link between paediatric UTI history and subsequent disease development, such as overactive bladder, IC/BPS, and adult RUTI incidence<sup>78,79</sup>, study of this population and prevention of infection in paediatric patients is likely to decrease lifetime incidence of other urinary diseases.

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## Chapter 4

### 4 Enterococcal production of neuroactive substances

#### 4.1 Introduction

Microbial endocrinology, the study of host-microbe interactions through neuroendocrine hormones<sup>1</sup>, is a relatively old field, dating back over 100 years<sup>2</sup>, yet the concept of bacteria acting as an exogenous source of neuroactive compounds is relatively recent<sup>3</sup>, particularly with regard to disease pathogenesis and pharmacology. Indeed, the study of psychobiotics, probiotics capable of producing neuroactive compounds<sup>4</sup>, as a pharmacological delivery vehicle is a rapidly developing research field.

Many researchers have suggested the gut microbiota functions as an endocrine organ in its own right that is capable of regulating host homeostasis both locally within the gastrointestinal tract and also at more distally located sites<sup>4-8</sup>. Notable studies include those assessing the microbiota content in lean and obese humans and mice, dysbiosis associated with the onset of diabetes, metabolic syndrome, and irritable bowel syndrome secondary to acute gastritis<sup>9-14</sup>. Yet while the role of the gut microbiota in homeostasis is on the way to being thoroughly characterised, no steps have yet been taken with respect to the urinary microbiota. Our research thus focuses on the ability of *Enterococcus faecalis*, a member of this bacterial community, to influence bladder homeostasis.

Enterococci are one of the most common causes of urinary tract infection (UTI) and asymptomatic bacteriuria (ABU)<sup>15-17</sup>, yet the virulence properties between UTI and ABU strains are similar<sup>18-20</sup>. Further, patients with recurrent UTI (RUTI) often have ABU between symptomatic episodes<sup>16</sup>, suggesting uropathogens may be capable of mediating the host response or sensation perception. Additionally, enterococcal UTI symptoms are often characterised as less severe than those of a “classical” *E. coli* UTI<sup>17,21,22</sup>. Given the role of lipopolysaccharide (LPS) in pain mediation of uropathogenic *E. coli* (UPEC) infection<sup>23</sup>, study of the Gram-positive *E. faecalis* bypasses this confounding factor. Further, presence of enterococci has been associated with urinary disease states, including interstitial cystitis/bladder pain syndrome (IC/BPS), neurogenic bladder and in

the majority of non-symptomatic patients with a history of RUTI, while absence is associated with health<sup>16,24-27</sup>.

There is a precedent for lactic acid bacteria to interact with the nervous system through production or utilisation of neuroactive substances, such as  $\gamma$ -aminobutyric acid (GABA)<sup>3,28,29</sup>. Production of GABA has been demonstrated for a variety of bacteria including, *Bifidobacterium* sp., *Enterococcus* sp., *Lactobacillus* sp., *Leuconostoc* sp., *Streptococcus* sp., *Listeria monocytogenes*, and *Pseudomonas aeruginosa*<sup>30-35</sup>. In bacteria, the functional role of GABA varies; for example, *L. monocytogenes* and *Lactobacillus brevis* produce GABA in response to acid stress<sup>36,37</sup>, while GABA is a quorum sensing molecule for *P. aeruginosa*<sup>38</sup>. In humans, GABA functions as the primary inhibitory neurotransmitter, is involved in antinociception, and has been documented to inhibit nerve-mediated detrusor contraction of isolated detrusor strips<sup>39-42</sup>.

In this chapter, through a targeted metabolomics approach using solid-phase microextraction coupled with liquid chromatography-tandem mass spectrometry (SPME-LC-MS/MS) we assessed the production of neuroactive compounds by *E. faecalis* *in vitro*. We hypothesised that *E. faecalis* can produce neuroactive compounds that block the host's perception of pain or reduce the frequency of bladder contractions, thereby allowing the organism to persist undetected. Our approach targeted compounds that had previously been detected in urine, produced by other bacterial species and others with known neuroactive abilities (Table 4-1).

**Table 4-1. Neuroactive metabolites for targeted metabolomics.**

<b>Common Name</b>	<b>LogP*</b>	<b>Molecular Weight</b>
2-arachidonylglycerol (2-AG)	5.65/5.41 P	378.5454
3-Methoxytyramine (3-MT) <sup>†</sup>	-0.08 E	167.205
Acetylcholine (ACh) <sup>†</sup>	-2.9/-4.2 E	146.2074
Agmatine <sup>†</sup>	-1/-1.2 P	130.1915
Androsterone	3.69 E	290.4403
Angiotensin-II (Ang-II)	-0.8/-5.3 P	1046.1786
Dopamine (DA) <sup>†</sup>	-0.98 P	153.1784
Epinephrine (EPI) <sup>†</sup>	-1.37 E	183.2044
Glycine	-3.21 E	75.0666
Histamine	-0.70 E	111.1451
L-aspartic acid (aspartate)	-3.89 E	133.1027
L-glutamic acid (glutamate)	-3.69 E	147.1293
L-lysine	-3.05 E	146.1876
L-phenylalanine	-1.38 E	165.1891
L-proline	-2.54 E	115.1305
L-serine	-3.07 E	105.0926
Melatonin	1.42/1.15 P	232.2783
N-arachidonoyl dopamine (NADA)	-2.9/-4.1 P	160.1711
Norepinephrine (NE) <sup>†</sup>	-1.24 E	169.1778
Progesterone	3.87 E	314.4617
Resveratrol	2.57/3.4 P	228.247
Serotonin (5-HT) <sup>†‡</sup>	0.21 E	176.2151
Synephrine	-0.45 E	167.205
Taurine <sup>†</sup>	-2.2/-2.6 P	125.147
Testosterone	3.32 E	288.4244
Thiamine	-2.1/-3.1 P	265.355
Tryptamine <sup>†</sup>	1.55 E	160.2157
Tyramine <sup>†</sup>	-0.14/0.68 P	137.179
β-phenethylamine (β-PEA)	1.41/1.39 E	121.1796
γ-aminobutyric acid (GABA) <sup>†</sup>	-3.17 E	103.1198
γ-hydroxybutyric acid (GHB)	-0.6/-0.51 P	103.0966

\*LogP values are differentiated as either predicted (P) or experimental (E) according to the Human Metabolome Database<sup>43-45</sup>.

<sup>†</sup>Metabolites were included in quantitative experiments.

<sup>‡</sup>Serotonin was not included in the exploratory metabolomics experiments.

## 4.2 Materials and methods

### 4.2.1 Bacterial strains and urothelial cell lines

Bacterial strains used throughout these experiments are listed in Table 4-2. Strains were routinely cultured without aeration at 37°C, in brain-heart infusion broth (BHI; Difco Laboratories Inc., Franklin Lakes, NJ, USA) for *E. faecalis* and Luria-Bertani (LB) broth for *E. coli*. Experiments were conducted with LB, BHI, BHI supplemented with 0.25% yeast extract (Difco; BHIYE), and Mclean's Artificial Urine (AU)<sup>46</sup>.

Urinary bladder epithelial cell lines were acquired from the American Type Culture Collection (ATCC); 5637's (ATCC HTB-9) were initially isolated from a 68-year-old male with grade II carcinoma, and T24's (ATCC HTB-4) were isolated from an 81-year-old female with transitional cell carcinoma. Urothelial cells were maintained in T75 flasks, in a 5% CO<sub>2</sub> tissue culture incubator at 37°C in Roswell Park Memorial Institute medium 1640 (RPMI; Gibco, Burlington, ON, CAN) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 2 mM glutamine (Gibco) and penicillin-streptomycin (Gibco) at 100 U/mL and 100 µg/mL respectively. Supplemented-RPMI without the addition of antibiotics will herein be referred to as infection media.

Cells were routinely passed when confluency reached greater than 80%. To pass cells, the monolayer was gently washed with warm phosphate buffered saline (PBS) followed by trypsinisation for 5 (T24s) or 10 (5637s) minutes at 37°C and 5% CO<sub>2</sub> by addition of 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco). Cells were dislodged with gentle tapping and trypsin inactivated by the addition of cell culture media. For cell maintenance, cell suspensions were reconstituted 10-fold in a new T75 flask with fresh supplemented-RPMI. For experiments, an aliquot of the cell suspension was mixed 1:1 (v/v) with 0.4% trypan blue (Invitrogen, Carlsbad, CA, USA) and 10 µL of sample mixture added to the chamber port of a Countess Cell Counting Chamber Slide (Invitrogen) for use with a Countess Automated Cell Counter (Invitrogen). Only viable cell counts were considered. Cell suspensions then underwent centrifugation at 3,000 rpm for 6 minutes, were reconstituted in infection media, and subsequently seeded to 24-well tissue-culture treated plates (Sarstedt, Nümbrecht, Germany) at  $1 \times 10^5$  cells/mL. Plates

were incubated as above until the monolayer reached confluency, at approximately 48 hours.

For SPME-LC-MS/MS, HPLC grade acetonitrile, methanol and N,N-dimethylformamide (DMF) were purchased from Caledon Labs (Halton Hills, ON, CAN). Non-sterile polypropylene 96-well deep well plates and polystyrene-divinylbenzene weak anion exchange (PS-DVB-WAX) 80  $\mu\text{m}$  particles were purchased from VWR International (Mississauga, ON, CAN); while the Oasis hydrophilic-lipophilic balanced (HLB) 30  $\mu\text{m}$  polymeric reversed-phase particles were supplied by Waters (Mississauga, ON, CAN). All metabolites were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of metabolites were prepared at a concentration of 1 mg/mL in methanol or water, depending on solubility properties, and stored at  $-20^{\circ}\text{C}$  according to the manufacturer's instructions.

**Table 4-2. Strains used in this study.**

<b>Strain</b>	<b>Initial Isolation</b>	<b>Source</b>
<i>E. faecalis</i>		
A256	Urine of patient with RUTI and urosepsis <sup>47</sup>	Our laboratory
1131	Uropathogen <sup>48</sup>	Our laboratory
1396	Female with UTI <sup>49</sup>	Our laboratory
23241	Urine of patient with pyelonephritis <sup>50</sup>	ATCC <sup>a</sup>
29212 <sup>b</sup>	Urine <sup>51</sup>	ATCC <sup>a</sup>
OG1X	Derivative of oral isolate 2SaR <sup>52</sup>	Our laboratory
19433 <sup>b,c</sup>	Documentation not available	ATCC <sup>a</sup>
<i>E. faecium</i>		
19434 <sup>c</sup>	Documentation not available	ATCC <sup>a</sup>

<sup>a</sup>American Type Culture Collection.

<sup>b</sup>Strain often used in quality control testing.

<sup>c</sup>Type strain<sup>53</sup>.

#### 4.2.2 Bacterial and urothelial co-culture

Bacteria were prepared for co-infections as follows; overnight broth cultures (stationary phase) were reconstituted 100-fold to infection media and incubated to mid-exponential phase (4-hours) at 37°C and 5% CO<sub>2</sub>. Exponential phase cultures were then diluted to a final concentration equivalent to a multiplicity of infection (MOI) of 10:1, 50:1, or 100:1 bacteria per host cell in appropriately supplemented infection media (co-culture). The MOI was calculated based on the urothelial cell count from a representative well. Cell counts were determined as described above.

Confluent monolayers were washed three times with warm PBS, followed by the addition of 500 µL of the prepared bacterial suspension. Co-cultures were incubated at 37°C and 5% CO<sub>2</sub>.

#### 4.2.3 GABase Assay

A commercial preparation of GABA transaminase (GABA-T) and succinate semialdehyde dehydrogenase (SSDH) from *Pseudomonas fluorescens* (GABase; Sigma-Aldrich) was prepared at a concentration of 2 mg/mL in 75 mM potassium phosphate buffer with 25% (v/v) glycerol (pH 7.2); stocks were stored at -20°C. Initial assays were completed according to the optimized method of Tsukatani *et al.*<sup>54</sup>; 80 mM Tris-HCl buffer (pH 9.0) with 750 mM sodium sulfate, 10mM dithiothreitol (DTT), 1.4 mM nicotinamide adenine dinucleotide phosphate (NADP<sup>+</sup>) and 2.0 mM  $\alpha$ -ketoglutarate. GABase was added to the reactions at a final concentration of 0.3 µg/µL. Reactions were kept on ice until optical density (OD) readings; the OD was read every 60 seconds at 340 nm for 60 minutes at 37°C using an Eon microplate spectrophotometer (BioTek, Winooski, VT, USA). The assay was modified by the addition of ethanolamine-*O*-sulphate (EOS), a GABA-T inhibitor, allowing for the distinction between GABA and succinate semialdehyde<sup>55</sup>. Stock solutions of assay components and GABA were stored at -20°C, except for DTT, which was made fresh for each experiment.



#### 4.2.4 SPME-LC-MS/MS

##### 4.2.4.1 SPME blade preparation

Stainless steel blades (length, 50 mm; width 2.5 mm; depth, 0.7 mm) were prepared for the SPME coating as follows; sonicated with concentrated HCl for 60 minutes, washed with nanopure water, and subsequently dried at 180°C for 30 minutes. The final 20 mm of the blades was coated in a 50:50 (w/w) mixture of PS-DVB-WAX and HLB in polyacetonitrile (PAN) solution using a flask-type sprayer with nitrogen gas source. This coating was selected based on the metabolite target list (Table 4-1). The PAN solution was prepared by dissolving 10% (w/w) PAN in DMF and heating at 90°C for 60 minutes. Coatings were thermally cured at 150°C for 5 minutes, and the coating procedure repeated to a final thickness of 80  $\mu\text{m}$ <sup>56,57</sup>.

##### 4.2.4.2 Exploratory metabolomics sample preparation

To determine the bacterial production of neuroactive substances *in vitro*, stationary phase *Enterococcus* sp. were diluted 1:100 in BHI, BHIYE, or LB, and cultures were incubated without aeration at 37°C for 3 or 24 hours. At the appropriate time, aliquots were centrifuged at  $12,000 \times g$  for two minutes at room temperature, and the supernatant stored at -80°C. To determine if enterococci could induce neuroactive substance production by urothelial cells, T24s were exposed to *E. faecalis* 1131 or 29212 at an MOI of 10, 50, or 100. Following a 60-minute incubation, the supernatant from three replicates was pooled, centrifuged and stored as above. This preliminary experiment was conducted with duplicates of the pooled co-culture samples and the 24- and 3-hour bacterial cultures.

##### 4.2.4.3 Quantitative metabolomics sample preparation

For quantitation of metabolites, stationary cultures of *E. faecalis* 1131, 1396, 23241, and A256 were diluted 1/100 to LB or AU in triplicate. Cultures were incubated at 37°C, without aeration to exponential phase, 3 hours for LB and 6 hours for AU. At the appropriate time, 1.8 mL of culture was transferred to an eppendorf, centrifuged at  $12,000 \times g$  for two minutes at room temperature, and 1.5 mL of the supernatant transferred to a fresh eppendorf. The supernatant was stored at -80°C until extraction.

Prior to centrifugation, an aliquot of each culture was plated on BHI agar to confirm culture purity. Media controls were handled under the same conditions as samples.

Co-culture experiments were completed using 6-well tissue culture treated plates with T24s and 5637s seeded at  $1 \times 10^5$  cells/mL. Exponential phase enterococci were added to washed urothelial cells at an MOI of 10 and the co-culture incubated for 1 or 4 hours. Urothelial cells that were washed and exposed to fresh media were included as controls. Further, all samples and controls, including the calibration curve, were prepared using the same batch of media to reduce experimental variance.

#### 4.2.4.4 High-throughput SPME

The manual Concept 96-blade SPME device was provided by Professional Analytical System (PAS) Technology (Magdala, Germany). The system consists of eight rows of blades, which are each composed of 12 thin film pins. The rows are held together by nine inter-blade holders, generating a 96-blade SPME system that functions as part of an autosampler<sup>56</sup>.

Prior to extraction, blades were preconditioned in ethanol and water at a ratio of 7:3 (v/v) for 60 minutes with 1000 rpm of agitation. The extraction step then consisted of 1 ml of room temperature sample at 1000 rpm for 60 minutes. All target metabolites had previously been shown to reach equilibrium with the coating by 60 minutes. The extraction was followed by a 20-second wash in 0.1% formic acid at 1000 rpm, which functions to remove the particulates and macromolecules from the coating surface. Desorption was then performed for 60 minutes in acetonitrile and water at a ratio of 1:1 (v/v) with agitation. The resulting solution was stored at -80°C until LC-MS/MS analysis. A second desorption was performed to clean the blades.

#### 4.2.4.5 LC-MS/MS conditions

Chromatographic separation was performed using a Discovery HS F5, 5cm x 4.6mm ID, 3µm particle size (Supelco, Bellfont, PA, USA) column (Courtesy of the University of Waterloo). Mobile phase A consisted of water/acetic acid at 99:1 (v/v) and mobile phase B consisted of acetonitrile/acetic acid at 99:1 (v/v); the flow rate was 200 µL/minute. The

gradient conditions were as follows; 100% of phase A from 0 to 3.0 minutes, increasing the linear gradient to 0% phase A from 3.0 to 25.0 minutes, and an isocratic hold at 10% phase A until 34.0 minutes. The column was then re-equilibrated to the starting phase over 6 minutes, with a 5-minute hold at 100% phase A. Thus, total run time was 45 minutes per sample.

Sample analysis was performed using an API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray source. A CTC PAL autosampler (LEAP Technologies, Carrboro NC, USA) injected 20  $\mu$ L of sample to the LC-MS/MS system. The MS/MS analysis was performed in positive mode under multiple reaction monitoring conditions. Analyst software (AB Sciex LP, Concord, ON, CAN) was used for data acquisition and processing.

#### 4.2.5 Urothelial gene expression

##### 4.2.5.1 RNA extraction and cDNA conversion

Following either a 1-hour or 4-hour incubation of 5637s with *E. faecalis* or *E. coli*, the supernatant was removed, and 500  $\mu$ L TRIzol reagent (Invitrogen) was added to the co-culture. Following a 5-minute incubation at room temperature, the lysate was homogenised by pipetting and transferred to an RNase-free 1.5 mL eppendorf. Cell lysates were stored at -20°C until processed using the PureLink RNA MiniKit (Invitrogen) as per the manufacturer's instructions.

In brief, 200  $\mu$ L of chloroform (Sigma-Aldrich) was added to the microcentrifuge tube and shaken vigorously, followed by an additional 500  $\mu$ L TRIzol reagent. The solution was incubated at room temperature for 3 minutes followed by centrifugation at 4°C and  $12,000 \times g$  for 15 minutes. The upper phase was transferred to a fresh microcentrifuge tube, followed by the addition of 100% ethanol (Sigma-Aldrich) at a 1:1 ratio. This mixture was then vortexed and transferred to a Spin Cartridge, which was centrifuged at room temperature and  $12,000 \times g$  for 15 seconds. The column was washed with Wash Buffer I. The product was washed twice with Wash Buffer II and eluted in 30  $\mu$ L RNase-free water. The resulting RNA was converted to complementary DNA (cDNA) using the

High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Unused RNA was flash frozen and stored at -80°C.

#### 4.2.5.2 Quantitative PCR

Pre-validated quantitative PCR (qPCR) primers were purchased from Sigma-Aldrich against a selection of adherens junction proteins and *TRPV4* (Table 4-3). Quantitative PCR experiments were completed using *Power* SYBR Green PCR Master Mix (Applied Biosystems) on the QuantStudio 5 Real-Time PCR System (Applied Biosystems). Initial data analysis and quality control assessment was completed using the QuantStudio Design and Analysis Software v1.4.1 (Applied Biosystems). Data is presented as relative quantification according to the  $\Delta\Delta C_T$  method, wherein vehicle treated 5637s were used as the comparator with *GAPDH*, *ACTB*, and *B2M* as endogenous controls.

#### 4.2.6 Statistical analysis

All experiments were completed in biological and technical triplicates, except the exploratory metabolomics experiment, which was completed in duplicate. Results in the figures are presented as mean per group  $\pm$  standard deviation. To test for significance in the quantitative metabolite experiments, we analysed the data using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons post hoc tests.

Urothelial relative gene expression analyses were completed using two-way ANOVA followed by Dunnett's multiple comparisons post hoc tests. Data analysis was completed using GraphPad Prism v7.0a for Mac OS X (GraphPad Software, San Diego, CA, USA).

**Table 4-3. Sigma KiCqStart\* primers used in this study.**

Gene Name	Gene symbol	PrimerPairID	Gene ID	RefSeqID	Exons	Species
<b>Endogenous controls</b>						
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	H_GAPDH_1	2597	NM_002046	9-10	Human
$\beta$ -actin	ACTB	H_ACTB_1	60	NM_001101	3-4	Human
$\beta$ 2-microglobulin	B2M	H_B2M_1	567	NM_004048	2-3	Human
<b>Targets</b>						
Transient receptor potential cation channel subfamily V member 4	TRPV4	H_TRPV4_2	59341	NM_001177428	9-10	Human
$\alpha$ -catenin	CTNNA1	H_CTNNA1_2	1495	NM_001903	6-7	Human
$\beta$ -catenin	CTNNB1	H_CTNNB1_1	1499	NM_001904	8-9	Human
$\gamma$ -catenin (junction plakoglobin)	JUP	H_JUP_1	3728	NM_002230	5-6	Human
E-cadherin	CDH1	H_CDH1_1	999	NM_004360	2-3	Human

\*PrimerPair ID identifies commercially available primers through Sigma-Aldrich.

## 4.3 Results

### 4.3.1 GABase assay

To measure GABA production by enterococci, we first needed to optimise an enzyme-based microtiter plate assay with GABA standards in LB. We were able to increase the final absorbance at 340 nm with various adaptations, including the addition of EOS to control for succinic semialdehyde in the sample, decrease in reaction temperature, and increase in reactants (Figure 4-1). Despite this improvement in absorbance, we were unable to achieve the sensitivity levels equivalent to urinary GABA concentrations, which ranges between 0.543 µg/mL (5.27 µM) and 67-179 ng/mL (0.65-1.7 µM)<sup>58,59</sup>.

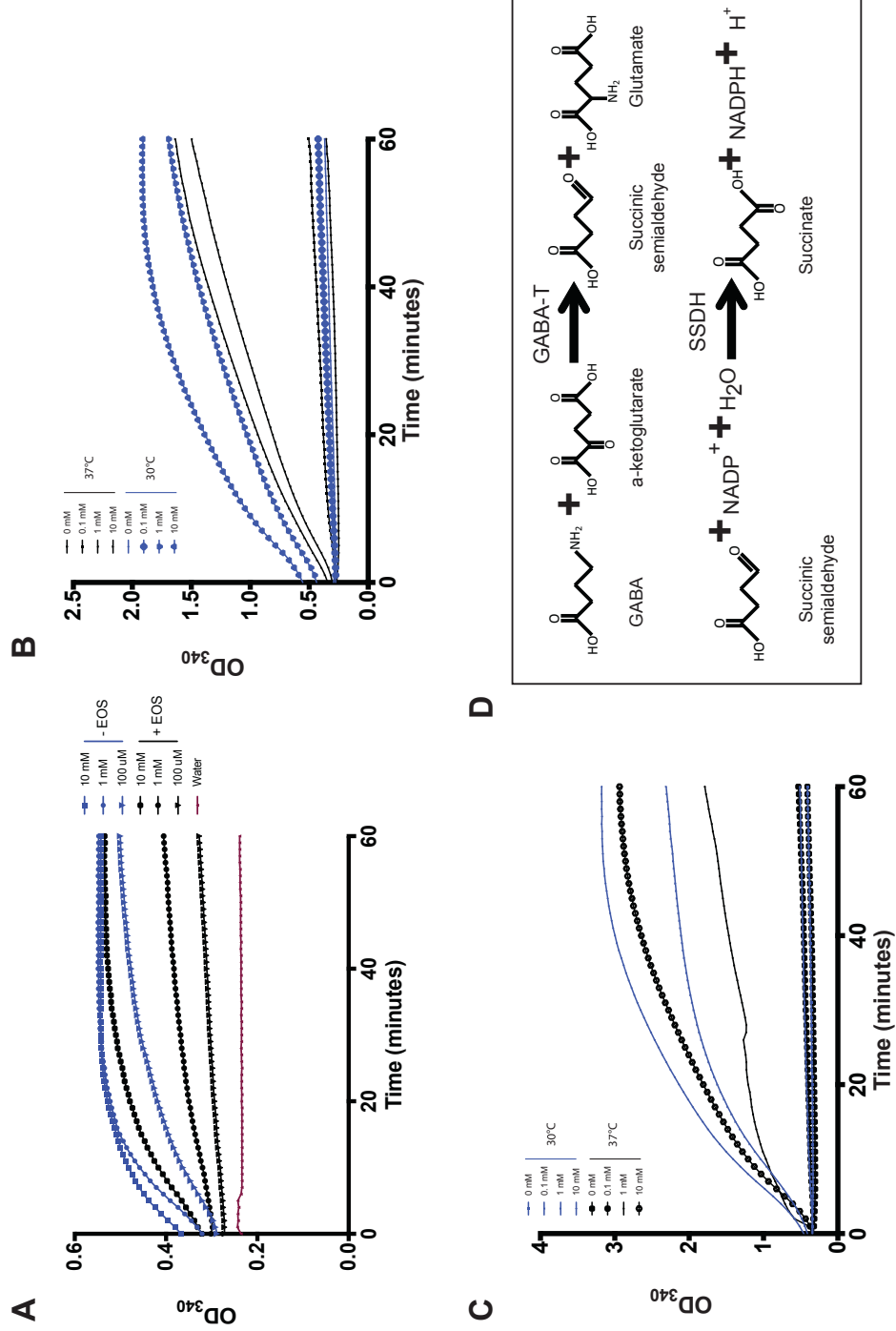
### 4.3.2 Exploratory SPME-LC-MS/MS

Given our issues with the lack of sensitivity of the GABase assay for our requirements, we altered our methodology to utilise a mass spectrometry approach which gave us the opportunity to target a variety of metabolites, rather than just one per assay (Table 4-2). In a preliminary screening analysis of our bacterial supernatants using the high-throughput LC-MS/MS, we elected not to include standard curve of various metabolites, nor did we include internal standards to maximum the number of samples that could be screened. Our aim was to narrow our metabolite target list, strains, and conditions for future experiments. Data was therefore analysed as the average change in analyte peak area relative to the media control. The results were not quantitative; however, we based our preliminary analysis on the assumption that a higher analyte peak area in the samples relative to controls correlated with bacterial and/or urothelial production of the metabolite of interest.

Bacterial production of tyramine was demonstrated in the supernatant of all *E. faecalis* strains cultured in LB, BHI, or BHYE at both 3 and 24 hours (Figure 4-2). *Enterococcus faecium* 19434 did not produce tyramine at 3 hours in BHYE and produced less tyramine than the *E. faecalis* strains at 3 hours in BHI and LB. Other biogenic amines, including, dopamine (DA), norepinephrine (NE), epinephrine (EPI), 3-methoxytyramine (3-MT), tryptamine, and histamine (HIS) were also detected at higher concentrations in samples

than media controls. Production of the amino acids proline, aspartate, glutamate, phenylalanine, and lysine was demonstrated under most conditions. Interestingly, a subset of samples appeared to be producing angiotensin-II (Ang-II), resveratrol, synephrine,  $\gamma$ -hydroxybutyric acid (GHB),  $\beta$ -phenethylamine ( $\beta$ -PEA), and GABA. Further, culture in LB was associated with production of a wider variety of metabolites than BHI or BHYE and profiles were generally quite similar between the 3 and 24-hour time points.

Co-culture of *E. faecalis* 29212 or 1131 with T24s was associated with production of proline, tyramine, glutamine, GHB, agmatine, tryptamine and acetylcholine (ACh) when compared to the supernatant of T24s that were not exposed to bacteria (Figure 4-3). Analyte peaks were generally lower in the co-cultures than pure cultures. Resveratrol, synephrine, DA, phenylalanine, and lysine were not produced by enterococcal-urothelial cell co-culture. Similarly, signals were detected from standards but not samples for; 2-arachidonoylglycerol (2-AG), N-arachidonoyl dopamine (NADA), testosterone, androsterone, progesterone, and melatonin.

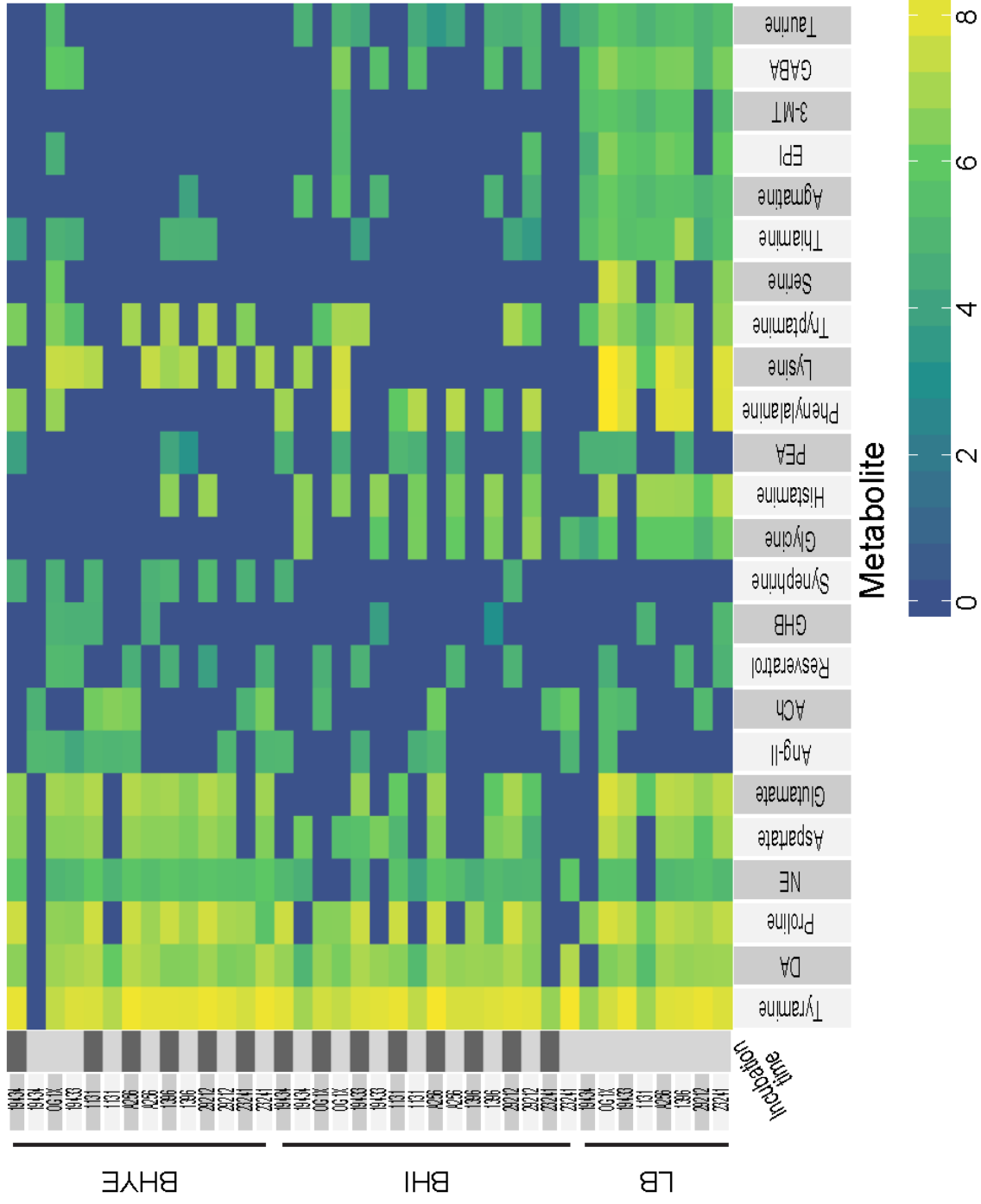


**Figure 4-1. GABase assay optimisation.**

**A** | The GABase assay was completed with 10, 1, and 0.1 mM GABA in LB, in the presence (black) and absence (blue) of ethanolamine-*O*-sulphate (EOS), water was included as a negative control. **B** | The GABase assay was run using GABA standards in LB at 30°C (blue) or 37°C (black). **C** | Concentrations of  $\alpha$ -ketoglutarate and  $\text{NADP}^+$  were increased to 4 mM and 3mM respectively



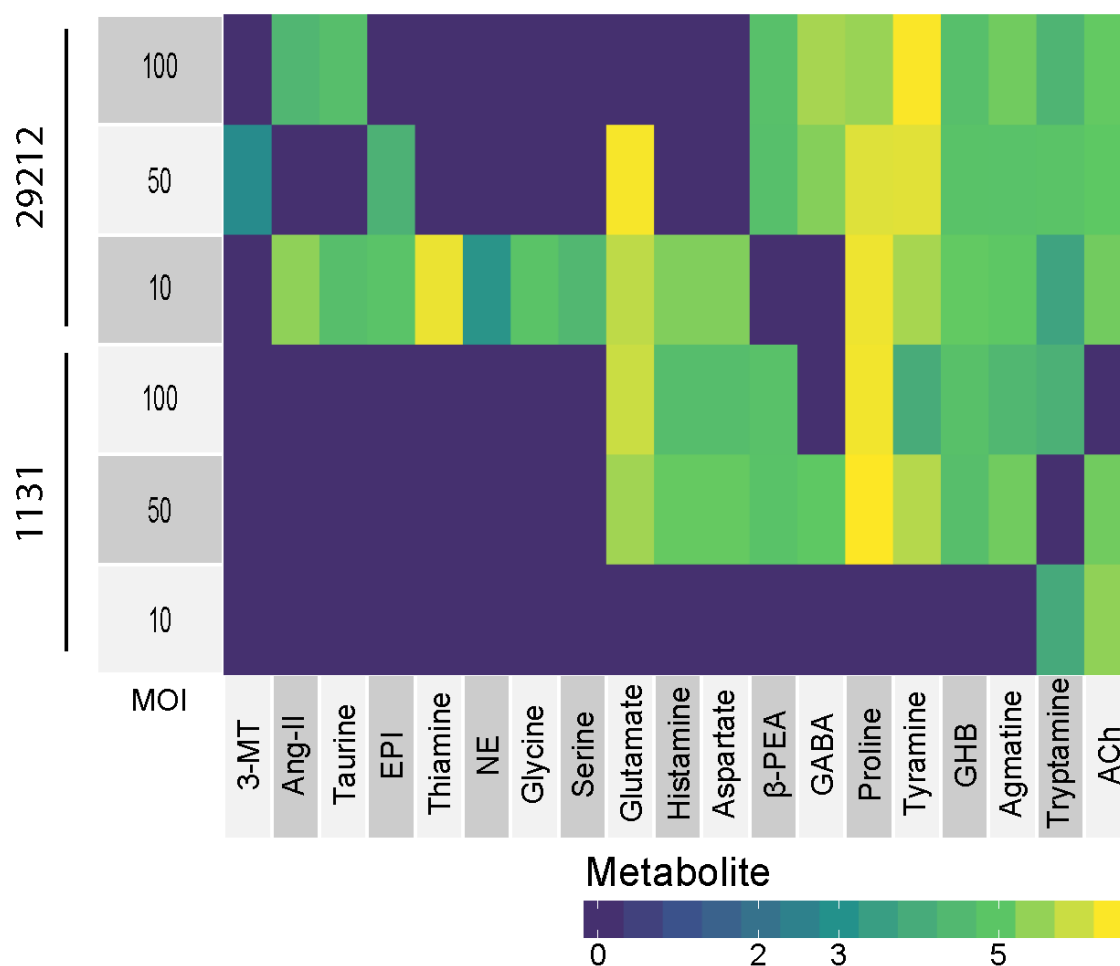
and the GABase reaction repeated at 37°C (black) and 30°C (blue). **D** | The GABase assay is dependent on the activity of two enzymes,  $\gamma$ -aminobutyric acid transaminase (GABA-T), which catalyses the reaction of GABA and  $\alpha$ -ketoglutarate to succinic semialdehyde and glutamate, and succinic semialdehyde dehydrogenase (SSDH), which converts succinic semialdehyde to succinate. Abbreviations: EOS, ethanolamine-*O*-sulphate; GABA-T,  $\gamma$ -aminobutyric acid transaminase; SSDH, succinic semialdehyde dehydrogenase. Graphs are representative of a single experiment with technical triplicates.



**Figure 4-2. Exploratory metabolite production by *Enterococcus* sp. *in vitro*.**

Metabolite production by seven strains of *E. faecalis* (23241, 29212, 1396, A256, 1131, 19433, and OG1X) and one *E. faecium* (19434) was measured via SPME-LC-MS/MS of the culture supernatant. Enterococci were incubated in LB (bottom), BHI (middle), or BHIYE (top) for 3 (incubation time, light grey) or 24 hours (incubation time, dark grey). Data is presented as the log<sub>10</sub> transformation of the change in analyte peak area relative to media control of two experiments. Only positive data is presented, relative decreases in analyte peak were considered to be 0 for this analysis.

Abbreviations: 3-MT, 3-methoxytyramine; ACh, acetylcholine; Ang-II, angiotensin-II; BHI, brain-heart infusion broth; BHIYE, BHI with 0.25% yeast extract; DA, dopamine; EPI, epinephrine; GABA,  $\gamma$ -aminobutyric acid; GHB,  $\gamma$ -hydroxybutyric acid; LB, Luria-Bertani broth; NE, norepinephrine;  $\beta$ -PEA, phenethylamine.



**Figure 4-3. Exploratory metabolite production by enterococcal-urothelial co-culture.**

Metabolites were measured via SPME-LC-MS/MS of the supernatant from a T24 monolayer exposed to *E. faecalis* 29212 or 1131 for 1-hour. Enterococci were added at a multiplicity of infection (MOI) of 10, 50, or 100 enterococci per T24 cell. Data is presented as the log<sub>10</sub> transformation of the change in analyte peak area relative to untreated T24s of two experiments. Only positive data is presented, relative decreases in analyte peak were considered to be 0 for this analysis.

Abbreviations: 3-MT, 3-methoxytyramine; ACh, acetylcholine; Ang-II, angiotensin-II; EPI, epinephrine; GABA, γ-aminobutyric acid; GHB, γ-hydroxybutyric acid; MOI, multiplicity of infection; NE, norepinephrine; β-PEA, β-phenethylamine.

### 4.3.3 Quantitative SPME-LC-MS/MS

Based on the results obtained through our exploratory SPME-LC-MS/MS run we opted to focus our efforts on enterococcal production of tyramine, tryptamine, taurine, agmatine, NE, EPI, 3-MT, GABA, DA, and ACh, as well as serotonin (5-HT). We restricted our enterococcal culture conditions to 3 hours in LB or AU and expanded the co-culture to include two cell lines at two time points. We defined the limit of detection (LOD) as 3 times the signal-to-noise (s/n) ratio and the limit of quantitation (LOQ) as 10 times the noise.

As such, we detected tyramine production by enterococci in AU up to 940  $\mu\text{M}$  (Table 4-4), and this was significantly higher in supernatant from *E. faecalis* 1396, 23241, and A256 ( $P = 0.001$ ) relative to the experimental control. We were unable to quantitate tyramine in LB cultures; however, tyramine was produced by *E. faecalis* 1396, 23241, and A256 and was not detected in the experimental LB controls or *E. faecalis* 1131 supernatant. In our co-culture experiments, the tyramine concentration was not significantly higher in supernatant from T24 or 5637 cells exposed to enterococci (Table 4-5), nor was there production of tyramine when *Enterococcus* was incubated in infection media alone. The data was also suggestive of enterococcal production of tryptamine; however, this trend was not statistically significant. Agmatine, EPI, GABA, NE, and taurine were not detected in any of the samples above the LOD. Similarly, ACh was only detected in AU and the supernatant from 5637 exposed to *E. faecalis* A256 for 1 hour, while DA was only detected in AU samples. Finally, 5-HT was detected in AU and co-culture supernatants, but not LB. Enterococcal culture in infection media, without urothelial cells, was included in the experiment to differentiate between enterococcal production of metabolites in response to urothelial cells or culture media; however, concentrations of metabolites were not significantly increased in samples relative to experimental blanks.

### 4.3.4 Urothelial response to bacteria

Given that the host response to bladder infection involves shedding of umbrella cells and transient receptor potential cation channel subfamily V member 4 (TRPV4) have been

linked with adherens junctions (AJs)<sup>60</sup>, we assessed the effect of enterococcal and *E. coli* infection on expression of AJ proteins and *TRPV4*. Expression of *TRPV4*,  $\alpha$ -catenin (*CTNNA1*),  $\beta$ -catenin (*CTNNB1*),  $\gamma$ -catenin (junction plakoglobin, *JUP*), and E-cadherin (*CDH1*) was unchanged following both the 1 and 4 hour incubations (Figure 4-4).

Table 4-4. Metabolite production by *E. faecalis*.

<i>E. faecalis</i> strain	Culture media	Metabolite (μM)			
		Tyramine*	Tryptamine	3-MT	DA
Bacteria free	LB	ND	0.460 (±0.237)	0.235 (±0.111)	ND
	AU	15.7 (±1.92)	0.803 (±0.586)	0.736 (±0.638)	0.517 (±0.375)
1131	LB	ND	0.649 (±0.732)	0.205 (±0.178)	ND
	AU	189 (±70.0)	0.424 (±0.726)	0.329 (±0.477)	0.527 (±0.480)
1396	LB	D	0.620 (±0.415)	0.223 (±0.154)	ND
	AU	838 (±273)	1.39 (±2.04)	0.554 (±0.887)	0.788 (±1.01)
23241	LB	D	0.321 (±0.094)	0.112 (±0.026)	ND
	AU	940 (±253)	0.274 (±0.383)	0.176 (±0.228)	ND
A256	LB	D	1.03 (±0.785)	0.294 (±0.209)	ND
	AU	813 (±186)	0.456 (±0.449)	0.192 (±0.149)	0.248 (±0.100)

\*Due to issues with the standard curve we were unable to quantitate tyramine in LB.

Abbreviations: 3-MT, 3-methoxytyramine; ACh, acetylcholine, AU, artificial urine; D, detected but not quantitated; DA, dopamine; LB, Luria-Bertani broth; ND, not detected.

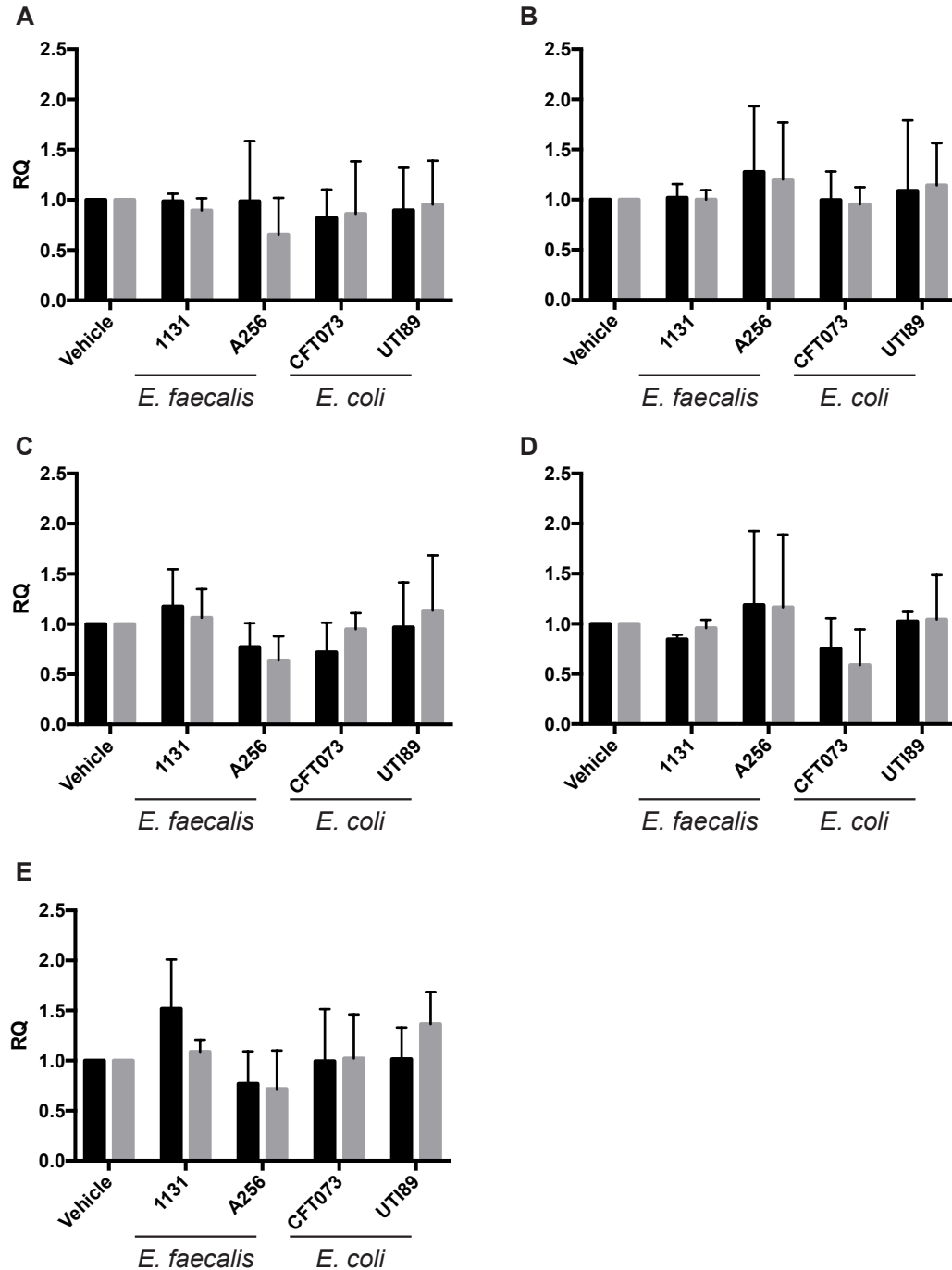
Table 4-5. Metabolite production by co-culture of *E. faecalis* and urothelial cells.

Cell culture conditions	Treatment	Metabolite ( $\mu\text{M}$ )			
		Tyramine	Tryptamine	Serotonin	3-MT
5637 – 1 hour	No treatment	3.02 ( $\pm 1.94$ )	3.01 ( $\pm 4.02$ )	0.232 ( $\pm 0.117$ )	0.112 ( $\pm 0.138$ )
	1131	4.35 ( $\pm 5.78$ )	ND	0.278 ( $\pm 0.180$ )	ND
	23241	4.12 ( $\pm 2.41$ )	3.63 ( $\pm 6.28$ )	0.355 ( $\pm 0.229$ )	0.115 ( $\pm 0.156$ )
	A256	3.67 ( $\pm 3.63$ )	2.48 ( $\pm 5.20$ )	0.236 ( $\pm 0.170$ )	0.048 ( $\pm 0.104$ )
5637 – 4 hours	No treatment	2.15 ( $\pm 0.736$ )	1.67 ( $\pm 1.78$ )	0.242 ( $\pm 0.148$ )	0.036 ( $\pm 0.039$ )
	1131	3.12 ( $\pm 1.47$ )	ND	0.221 ( $\pm 0.050$ )	ND
	23241*	-	-	-	-
	A256	2.27 ( $\pm 2.35$ )	1.47 ( $\pm 0.783$ )	0.204 ( $\pm 0.143$ )	0.004 ( $\pm 0.012$ )
T24 – 1 hour	No treatment	2.48 ( $\pm 0.437$ )	ND	0.200 ( $\pm 0.113$ )	ND
	1131	2.23 ( $\pm 0.780$ )	0.569 ( $\pm 0.298$ )	0.230 ( $\pm 0.051$ )	0.007 ( $\pm 0.028$ )
	23241	4.23 ( $\pm 5.06$ )	ND	0.178 ( $\pm 0.115$ )	ND
	A256	2.99 ( $\pm 2.51$ )	ND	0.205 ( $\pm 0.133$ )	ND
T24 – 4 hours	No treatment	2.40 ( $\pm 0.437$ )	0.213 ( $\pm 0.074$ )	0.219 ( $\pm 0.088$ )	ND
	1131	3.06 ( $\pm 1.60$ )	2.04 ( $\pm 3.19$ )	0.100 ( $\pm 0.105$ )	0.062 ( $\pm 0.125$ )
	23241*	-	-	-	-
	A256	2.25 ( $\pm 2.81$ )	1.06 ( $\pm 1.24$ )	0.221 ( $\pm 0.098$ )	0.003 ( $\pm 0.020$ )
Infection media – 1 hour	No treatment	6.06 ( $\pm 5.82$ )	0.833 ( $\pm 0.781$ )	0.257 ( $\pm 0.098$ )	0.023 ( $\pm 0.050$ )
	1131	6.39 ( $\pm 2.32$ )	ND	0.084 ( $\pm 0.020$ )	ND
	23241	2.85 ( $\pm 2.62$ )	0.872 ( $\pm 0.191$ )	0.163 ( $\pm 0.100$ )	ND
	A256	5.81 ( $\pm 5.36$ )	1.03 ( $\pm 0.889$ )	0.205 ( $\pm 0.171$ )	0.014 ( $\pm 0.039$ )
Infection media – 4 hours	No treatment	1.95 ( $\pm 0.977$ )	1.61 ( $\pm 1.91$ )	0.179 ( $\pm 0.086$ )	ND
	1131	1.89 ( $\pm 1.49$ )	2.61 ( $\pm 3.79$ )	0.229 ( $\pm 0.115$ )	0.021 ( $\pm 0.067$ )
	23241*	-	-	-	-
	A256	3.46 ( $\pm 5.12$ )	0.761 ( $\pm 0.877$ )	0.286 ( $\pm 0.196$ )	0.014 ( $\pm 0.041$ )

\**E. faecalis* 23241 was not included in the 4-hour experiments.

Abbreviations: 3-MT, 3-methoxytyramine; ND, not detected.





**Figure 4-4. Urothelial gene expression of adherens junction proteins in response to bacterial infection.**

Urothelial gene expression of adherens junction proteins was assessed by qPCR following either a 1-hour (black bars) or 4-hour (grey bars) exposure to *E. faecalis* (1131 or A256) or *E. coli* (CFT073 or UT189). Expression of *TRPV4* (A), *CTNNA1* (B), *CTNNB1* (C), *JUP* (D), and *CDH1* (E) is presented relative to vehicle controls.

Experiments and qPCR reactions were completed in triplicate. Means from at least three different experiments are shown with significance. Significance was determined against the vehicle control using two-way ANOVA with Dunnett's multiple comparisons test.

## 4.4 Discussion

Given the recent studies demonstrating the links between oral administration of GABA-producing lactic acid bacteria with resultant decreases in anxiety and depression-like symptoms in mice<sup>3,4,61</sup>; we postulated that enterococci produce neuroactive substances in the bladder. These metabolites, in turn, would decrease bladder contractions, which are associated with urgency and frequency, and host perception of pelvic pain and/or dysuria. Initial experiments targeted only GABA; however, we transitioned to mass spectrometry methods to increase sensitivity, which also corresponded with expansion of the target list. The application of SPME-LC-MS/MS to enterococcal cultures indicated a variety of metabolites were being produced. Using a refined list of target metabolites, we then quantified the enterococcal production of tyramine at concentrations up to 940  $\mu$ M in AU.

Approximately 5% of patients meeting the laboratory diagnostic criteria for UTI are symptom-free - by definition, these patients have ABU<sup>62</sup>. The molecular mechanisms responsible for the differences in these two states are not understood, although it is well documented that ABU isolates demonstrate similar virulence properties to UTI isolates<sup>18-20</sup>. The traditional assumption is that UTI-associated pain is a consequence of bladder inflammation; however, a murine model of UPEC UTI indicates this is not the case<sup>23</sup>. Using this model, researchers were unable to correlate bacterial load or inflammation with pain. Rather, LPS-activation of toll-like receptor 4 induced pain independent of neutrophil-induced inflammation. Patients with ABU, who by definition are not symptomatic, often have pyuria<sup>62,63</sup>, indicating that inflammation alone is not sufficient to mediate pain. This change in our understanding of pain modulation in UTI raises interesting avenues of exploration. Indeed, if UPEC-associated pain is not correlated with bacterial load or inflammation, but is induced by LPS, how do Gram-positive bacteria, such as *E. faecalis*, induce bladder pain and how do they mitigate the symptoms of UTI?

Contrary to popular belief, many classical neurotransmitters and neuromodulation systems are found outside of the nervous system in eukaryotes. Indeed, communication between the nervous and immune system is dependent on many such factors. For example, Substance P is the primary pain signal in the nervous system, yet also

modulates inflammation within the immune system<sup>64</sup>. This highlights a key interplay between these two systems in infectious states; signalling to the brain that there is something wrong (pain) and initiating the immune response to foreign invasion through recruitment of neutrophils<sup>65</sup>. While neuroactive substances are primarily active within the central, peripheral, and enteric nervous systems, there is plenty of research suggesting such molecules are active in other tissues as well. Many receptors involved in neural transmission are present, and active, within non-neuronal tissues of the bladder<sup>66-70</sup>.

The initial experimental design involved the application of an enzyme-based microtiter plate assay for the measurement of GABA produced by enterococci. This method is dependent on the “in series” enzymatic reactions of GABA-T and SSDH (GABase), of which the final products are succinate, NADPH, and  $H^+$  (Figure 4-1, D). Absorbance is measured at 340 nm, and the NADPH-mediated increase in absorbance is assumed to directly correlate with the concentration of GABA in the sample<sup>54</sup>; however, this does not account for any succinic semialdehyde present in the sample and could lead to inaccurate results (data not shown). To overcome this, we repeated our experiments, using GABA standards in LB with EOS, an inhibitor of GABA-T (Figure 4-1, A)<sup>55</sup>. As expected, the addition of EOS to the GABase reaction decreased absorbance and provided a method to measure the contribution of succinic semialdehyde to the reaction. We then moved forward with reaction optimisation as the change in absorbance between negative controls, such as water, and 10 mM GABA was less than 0.4 in our standard runs.

Reaction optimisation included; decreasing the temperature to 30°C, replacing the reducing agent (DTT) with  $\beta$ -mercaptoethanol, incorporation of 0.1 M EDTA to stabilise DTT, and increases in the reactants,  $\alpha$ -ketoglutarate and  $NADP^+$  from 2 and 1.4 mM, respectively, to 4 and 3 mM. Given that optimal growth conditions for *P. fluorescens*, which is the source of the GABase, are between 25°C and 30°C, the inverse relationship between absorbance and temperature was expected (Figure 4-1, B). Increases in  $\alpha$ -ketoglutarate and  $NADP^+$  were also associated with increased absorbance (Figure 4-1, C). For measurement of GABA, it is key that other reactants, such as  $\alpha$ -ketoglutarate and  $NADP^+$  are present in the reactions in excess and that GABA is the limiting factor. Unfortunately, despite numerous attempts and experimental permutations, we were

unable to achieve assay sensitivity equivalent to urinary GABA concentrations. Indeed, we were unable to detect GABA below 100  $\mu\text{M}$  and urinary GABA has been measured in the range of 0.543  $\mu\text{g/mL}$  (5.27  $\mu\text{M}$ ) and 67-179  $\text{ng/mL}$  (0.65-1.7  $\mu\text{M}$ )<sup>58,59</sup>.

Given our desire for a more sensitive method, we transitioned to LC-MS/MS and increased our target list (Table 4-1). The target list was developed based on a thorough review of the literature for neuroactive substances that had previously been documented as produced by bacteria, were known to have effects on the bladder or urothelium, or had been detected in human urine. Notably, Table 4-1 does not include all of the molecules identified through this search, but rather a subset of the list based on compound availability, extraction using SPME blades coated with PS-DVB-PAN:HLB (50:50), and identification by LC-MS/MS. Given its high-throughput nature, requirements for small sample volume and high sensitivity<sup>71</sup>, we selected SPME as the extraction method. Further, our collaborators had recently demonstrated quantitation of DA, GABA, 5-HT, and glutamate by SPME in the range of 20-35  $\text{pg/mL}$  from rat brain tissue and synthetic cerebral spinal fluid, as well as successfully applied SPME to *E. coli* metabolomics<sup>57,72,73</sup>.

The initial SPME-LC-MS/MS experiments were conducted primarily to narrow our research focus. Thus we completed these experiments without an extraction standard curve or internal standards. While this meant we sacrificed quantitative capabilities, we were able to analyse more conditions, which was key to identifying *Enterococcus* strains for further experimentation. This significantly limited the conclusions we could take from the data; however, we assumed that a higher analyte peak area in samples relative to controls correlated with production of the metabolite of interest and used this as a baseline for later studies.

Based on these assumptions, the data is indicative of *E. faecalis* production of tyramine under most of the conditions assayed. This was expected as tyramine production, through the decarboxylation of tyrosine, is a defining characteristic of *E. faecalis*<sup>74-84</sup>. Enterococci also appeared to be producing DA, NE, EPI, 3-MT, histamine (HIS), and tryptamine under most conditions, and resveratrol, synephrine, GHB, Ang-II,  $\beta$ -PEA, and GABA in a subset of samples. In addition to tyramine, *E. faecalis* production of agmatine, DA,  $\beta$ -

PEA 5-HT, tryptamine, and HIS has previously been documented<sup>77-86</sup>. Interestingly, culture in LB was associated with the production of a wider variety of metabolites than BHI or BHYE, which may be a reflection of nutrient availability, as LB is the least nutrient-rich of the three media. Enterococcal co-culture with T24s suggested enterococci produced, or induced host-production of, proline, glutamine, GHB, ACh, tyramine, tryptamine, and agmatine. Interestingly, the analyte peaks were generally lower in the co-cultures than pure cultures, which may be a result of a shorter incubation time (1 vs 3 or 6 hours), a measure of the enterococcal response to FBS, or linked to the increased nutrient value. That this decreased signal is indicative of the urothelial response to abnormal metabolite concentrations is unlikely, as *E. faecalis* growth in infection media was assessed in the quantitative experiments and demonstrated a similar trend.

In contrast, 2-AG, NADA, testosterone, androsterone, progesterone, or melatonin were not detected; however, we cannot rule out bacterial production of these analytes as we did not include extraction controls, and the method had not been assessed for extraction from infection media at this time. Method validation in infection media and AU was completed at the time of quantitative metabolomics. Further, lack of signal may indicate that enterococci do not produce these compounds under these culture conditions, that they were produced below the limit of detection, or that they failed to bind the extraction interface in the complex media.

Accordingly, we narrowed our metabolite list for further study to; DA, NE, EPI, 3-MT, 5-HT, ACh, tyramine, taurine, tryptamine, and agmatine (Table 4-1). Based on the higher occurrence of neuroactive molecules in the LB samples, we opted to repeat the experiments using LB and AU, which are significantly less nutrient-rich than BHI and BHYE. Interestingly, the quantitative metabolomics experiments demonstrated significant enterococcal production of tyramine. For example, *E. faecalis* 23241 produced 940  $\mu$ M in AU, which correlates to between approximately 190 and 3000 times the average concentration of tyramine in human urine<sup>87-89</sup>. Due to issues with the standard curve, we were unable to quantitate tyramine in LB.

Tyramine production by *E. faecalis* is an acid resistance mechanism and has been suggested to contribute to supplemental energy acquisition<sup>90-92</sup>. This may explain why we were able to measure tyramine production in AU, but not infection media, as the expression of tyrosine decarboxylase, which catalyses the formation of tyramine, is 10-fold upregulated at pH 5 relative to pH 7<sup>91</sup>. For perspective, the pH of AU is 5.8<sup>46</sup>, while the pH of infection media is 8.2.

Given that most studies on enterococcal production of tyramine are completed from a food science perspective, little information is available about the implications of microbial tyramine production in humans or how this contributes to endogenous tyramine concentrations. Interestingly, *in vitro* study of *Enterococcus durans* has demonstrated enterococcal production of tyramine under conditions stimulating gastrointestinal transit and linked both immunomodulation of, and increased attachment to, enterocytes via tyramine production<sup>93</sup>. This mechanism of tyramine-induced attachment to the gastrointestinal luminal surface has been shown for multiple bacterial species<sup>94-97</sup>. The concentrations of tyramine assessed in these studies (10 - 1000  $\mu$ M) correlates with the concentrations we determined in AU, suggesting this may be a mechanism by which enterococci promote polymicrobial UTI.

In humans, tyramine is recognised as a neuromodulator and has been shown to modulate GABA, ACh, DA, 5-HT, and NE signalling through activation of trace amine-associated receptor (TAAR) 1 and 2<sup>98-101</sup>. To the best of our knowledge, no studies have assessed the impact of intravesical instillation of tyramine or TAAR1/2 agonists, yet TAAR1 is highly expressed in the urothelium<sup>102,103</sup>. Study of TAARs is compounded by a number of factors, including low basal expression levels, lack of sensitive and specific human TAAR antibodies, lack of a single consensus ligand, and high inter-species variability and ligand promiscuity<sup>100,101,104-106</sup>. No data is available on TAAR2 expression in the bladder.

Although most tyramine research has focused on its neuromodulatory functions, the study of tyramine-induced detrusor strip contraction suggests the contractile action may be nonadrenergic in guinea-pigs and rabbits<sup>107,108</sup>. With regard to human health, levels of

tyramine in cheese have been extensively studied as ingestion of high concentrations can trigger hypertensive crises in people taking monoamine oxidase (MAO)-inhibitors, an effect that is mediated through the increased release and inhibited reuptake of NE, DA, and 5-HT<sup>90,109</sup>. Further, urinary tyramine is increased in IC/BPS patients relative to healthy controls, and increased tyramine concentrations have also been correlated with diseases characterised by pain, such as migraine<sup>110,111</sup>.

Co-culture samples were not associated with increased production of tyramine, or any of the metabolites analysed. Further, while the data was suggestive of tryptamine production, this was not significantly higher in samples relative to media controls. Indeed, our quantitative results present an interesting contrariety; the noise was determined as the average analyte peak area of matrix blanks and the LOD of detection as three times the s/n ratio, yet experimental blanks, which were handled alongside samples, gave signals significantly higher than the LOD, some of which were above the LOQ. All experimental blanks were confirmed as sterile prior to storage at -80°C, thus, explaining this discrepancy as contamination is unlikely. Further, all matrix blanks and samples were from the same media batch. Thus variance due to media preparation can also be ruled out. It is possible that the storage conditions induced breakdown of the metabolites in the matrix blanks, thus artificially reducing the s/n ratio. Alternatively, the metabolites may be present at such low concentrations, that noise and signal are indistinguishable by this methodology.

Prior to the exploratory metabolomics experiments, the SPME-LC-MS/MS method was validated in terms of linearity, sensitivity, LOD and LOQ, in PBS and LB, wherein the LOD and LOQ were in the range of 0.1-3 and 0.4-9 ng/mL, respectively. However, the method was not validated for BHI, BHIYE, or infection media, which may explain some of the discrepancies between the exploratory and quantitative experiments. Further, the exploratory experiments were assessed as the relative increase in analyte peak area relative to the appropriate control, not as a factor of the s/n ratio.

To measure the bladder's response to tyramine, and the other metabolites, we obtained a human bladder smooth muscle cell line (T4091; Applied Biological Materials Inc.,

Richmond, BC, CAN), which had previously been reported to contract in response to the muscarinic agonist bethanechol<sup>112</sup>. The authors of the original report, and the company, stated that these cells maintained contractile and smooth muscle cell properties beyond 50 passages. However, this was not the case, as the cells did not contract in response to bethanechol (data not shown) and this was later confirmed by the supplier. Thus, we transitioned to the measurement of the urothelial response to bacterial metabolites, using a commercial fluorescent calcium assay, originally been acquired to measure calcium influx of the contracting the smooth muscle cells. Unfortunately, downstream effects for most, if not all, of our metabolite targets involved calcium influx (data not shown), indicating this assay lacked specificity for our purposes without the inclusion of specific agonists or antagonists.

Finally, we assessed the potential for enterococci to differentially regulate the expression of *TRPV4* and adherens junction (AJ) proteins *in vitro*. Urothelial expression of the transient receptor potential (TRP) superfamily of ion channels has been well characterised<sup>113,114</sup>, and activation of TRPV4 by mechanical, chemical, and osmotic stimuli, such as cell swelling, shear stress, and anandamide, increases intracellular  $\text{Ca}^{2+}$ <sup>115,116</sup>. Urothelial co-localisation experiments demonstrate that TRPV4 interacts with AJs through  $\alpha$ -catenin and that this complex is located between umbrella cells in human and murine bladders<sup>60</sup>. In brief, the C-terminus of TRPV4 is coupled to  $\alpha$ -catenin, which interacts directly with the actin cytoskeleton and indirectly with E-cadherin through  $\beta$ - or  $\gamma$ -catenin (Figure 4-5). It has been proposed that this is a mechanism by which TRPV4 responds to bladder stretch<sup>60</sup>.

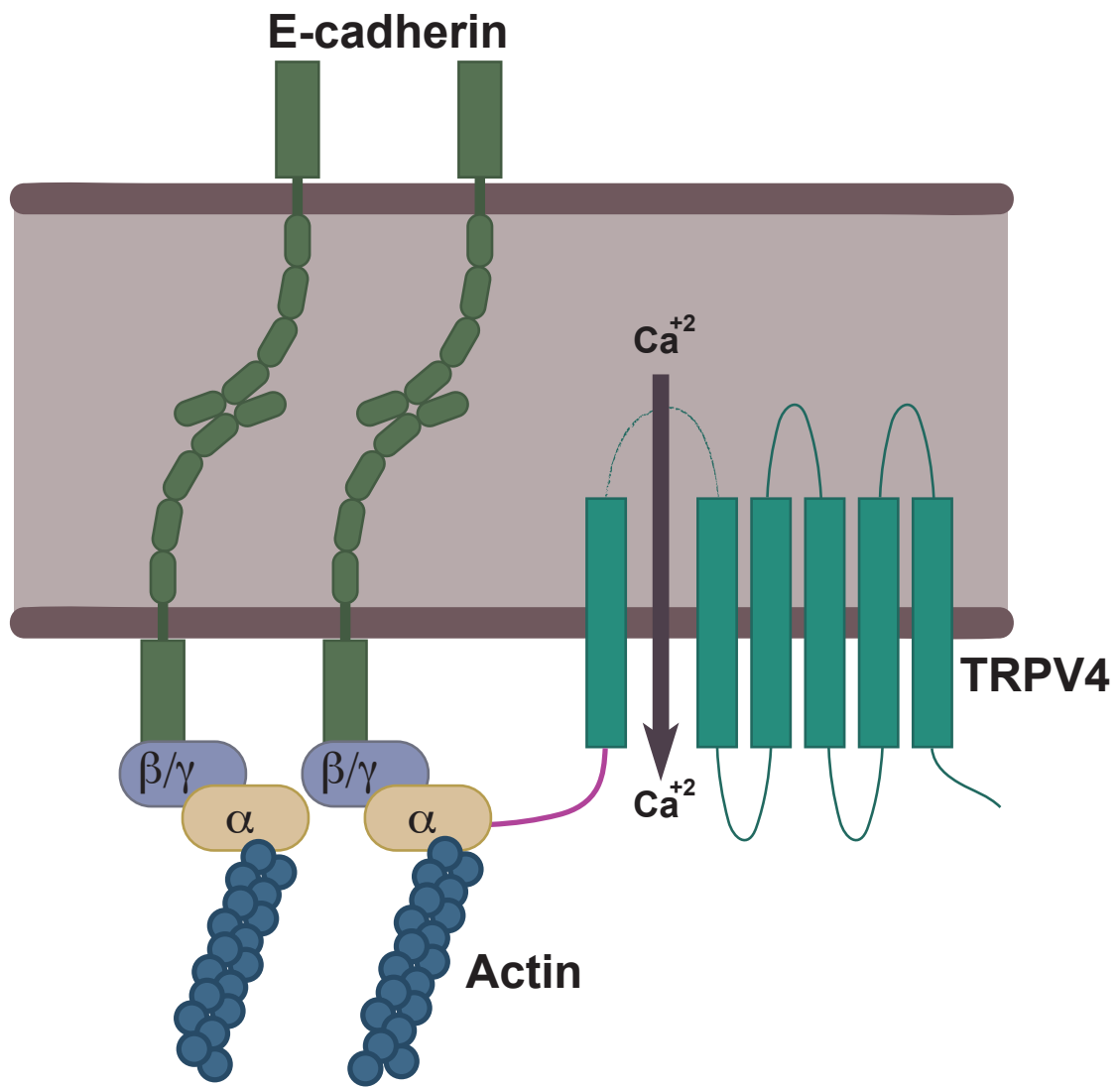
Interestingly, cyclophosphamide-induced cystitis in rats is associated with decreased *TRPV4* expression within the urothelium and detrusor 4 hours post-treatment, yet in a chronic model, *TRPV4* expression was increased<sup>113</sup>. To determine if bacteria could elicit a similar response, we exposed 5637s to *E. faecalis* or *E. coli* for 1 or 4 hours and measured relative gene expression. However, we did not note changes in relative expression for any of the genes assayed. Unlike our quantitative metabolomics experiments, which used both T24 and 5637s, these experiments were only completed



with 5637s. T24s do not express E-cadherin,  $\alpha$ -,  $\beta$ -, or  $\gamma$ -catenin, while 5637s express all four AJ proteins<sup>117</sup>.

#### 4.4.1 Conclusions

In conclusion, tyramine is produced by enterococci cultured in AU at concentrations significantly higher than encountered in the urine of healthy individuals. Further, TAAR1 is expressed within the urothelium, indicating that the bladder is capable of responding to bacterial production of this metabolite. We attempted to measure the urothelial response to tyramine, and the other target metabolites, through a number of avenues, including metabolite-induced or inhibited contraction of a human bladder smooth muscle cell line *in vitro* and visualisation of calcium influx in urothelial cells. However, the commercially available smooth muscle cells had lost their contractile properties, and calcium influx as an assay of urothelial cells lacks specificity without the inclusion of specific agonists or antagonists. Thus, the urothelial response to enterococcal tyramine remains to be demonstrated. Further, while, chemically induced cystitis disrupts *TRPV4* expression within the urothelium in a rat model, the same cannot be said for bacterial interactions with urothelial cells *in vitro*.



**Figure 4-5. Hypothetical model of TRPV4 interactions with adherens junctions.**

The TRPV4 response to bladder stretch is believed to be mediated through interactions with adherens junctions (AJs), leading to Ca<sup>2+</sup> influx. The C-terminus of TRPV4 is coupled to α-catenin, which interacts directly with the actin skeleton and indirectly with E-cadherin via either β- or γ-catenin. This figure has been modified from Janssen *et al.*<sup>60</sup>.

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## Chapter 5

### 5 General discussion

Urinary tract infection (UTI) pathogenesis has been extensively characterised with regard to *Escherichia coli*; however, non-*E. coli* UTI receives much less attention. This is understandable given that *E. coli* is the aetiological agent in the vast majority of UTIs. In contrast, *Enterococcus faecalis* is the least likely uropathogen to meet standardised diagnostic criteria<sup>1</sup>. In addition to monomicrobial UTI, *E. faecalis* is a major player in polymicrobial UTI<sup>2-5</sup>, wherein enterococci increase the virulence and reduce host clearance of other bacterial species<sup>6-9</sup>. However, polymicrobial UTI is likely under-diagnosed, as most polymicrobial urine samples are considered contaminated. This fact, in combination with the decreased likelihood of enterococcal infections meeting diagnostic criteria, suggests enterococcal UTI may often be missed.

The purpose of this thesis was to increase understanding of enterococcal UTI, in particular the response of *Enterococcus* to antibiotic prophylaxis *in vitro* and *in vivo* and enterococcal communication with the bladder. Within this thesis we demonstrated increased enterococcal virulence in response to growth inhibitory concentrations of nitrofurantoin *in vitro*, but not trimethoprim-sulfamethoxazole (TMP/SMX). This increased virulence did not correlate with increased expression of virulence factors, but was correlated with increased expression of three putative genes. We then explored whether this corresponded to alterations in bacterial communities throughout antibiotic prophylaxis for paediatric patients with recurrent UTI (RUTI). Our bacterial culture results indicated uropathogens were present in the urine of children with and without a history of RUTI and that antibiotic prophylaxis induced a transient decrease in uropathogen load. Interestingly, none of our patients were experiencing symptomatic UTI at the time of urine sample collection, yet a significant proportion of midstream (MS) urine samples met the clinical threshold for UTI, indicating these patients had asymptomatic bacteriuria (ABU). Further, *E. faecalis* bacterial load was positively correlated with non-*E. coli* uropathogens, suggesting some patients may be pre-disposed to polymicrobial UTI. In an effort to elucidate mechanisms by which enterococci can

mask the host's perception of UTI, we completed targeted metabolomics of neuroactive molecules *in vitro* under conditions mimicking the bladder environment. Our results suggest *Enterococcus* may produce tyramine in the bladder at concentrations that are likely to have a physiological effect on both urothelial cells and cohabiting bacteria, potentially leading to the development of asymptomatic infections, and prevalence of polymicrobial UTI commonly observed with *E. faecalis* infection.

A main tenet of this thesis was to assess whether antibiotic prophylaxis is an appropriate and effective therapeutic strategy for the treatment and prevention of RUTI. The increase in antimicrobial resistance trends, especially among *Enterococcus* sp., is concerning and indicates unnecessary antibiotic use should be restricted. Therefore, we assessed the effect of nitrofurantoin and TMP/SMX on bacterial attachment to urothelial cells in **Chapter 2**. Attachment is the first step to successful bacterial colonization of the bladder, and prevention or downregulation of this virulence trait will presumably decrease bacterial colonization rates and subsequent development of UTI. Coincidentally we identified a significant increase in the attachment of both *E. faecalis* and *E. coli* to urothelial cells at the minimal inhibitory concentration (MIC) of nitrofurantoin, but not TMP/SMX. To understand this phenotype better, we assessed the gene expression of classical *E. faecalis* adhesion and colonization factors, along with other genes that had been previously documented as differentially regulated under antibiotic stress or by growth in pooled human urine relative to laboratory growth medium<sup>10-13</sup>.

Contrary to the expected results, nitrofurantoin exposure decreased the expression of adhesion and colonization factors. Notably, we also documented an increase in the relative expression of two putative MDR efflux pumps, EF1732 and EF1733, as well as a putative DNA repair exonuclease, EF0972. Given that increased relative gene expression has been previously demonstrated for these loci in response to erythromycin and chloramphenicol<sup>11,12</sup>, two antimicrobials with different mechanisms of action compared to nitrofurantoin, we can assume that these gene products may be involved in either antibiotic-associated stress, or more general stress responses. Therefore, we believe EF1732, EF1733, and EF0972 may represent potential new antibiotic targets, particularly for use in tandem with the aforementioned antimicrobials. Application of such a

therapeutic strategy may decrease the rate of antibiotic resistance development, prolonging the clinical lifespan of these drugs.

The observation of increased enterococcal attachment in the presence of MIC nitrofurantoin is concerning and was a driving force behind the clinical study described in **Chapter 3**. The pilot study we conducted recruited less children receiving nitrofurantoin ( $n = 7$ ) than we had hoped; the remainder received TMP/SMX or were clinically observed. However, we were able to demonstrate the presence of bacteria in urine regardless of antibiotic status and in the urine of both healthy and RUTI patients. Despite the fact that none of the patients were symptomatic at the time of urine collection, 29% of MS urine samples from RUTI patients were indicative of ABU, supporting research that suggests patients experience periods of ABU between symptomatic episodes<sup>14</sup>.

We collected both first-void (FV) and MS urine from our study population. Clinically, MS urine is used to assess bacterial load in the bladder, while FV urine gives an indication of urethritis and is used for testing of some sexually transmitted infections, such as *Chlamydia trachomatis*<sup>15</sup>. As our interest lay within profiling the bacterial communities of the urinary tract we included both of these samples in our analysis. Additionally, we did not require patients to clean the labia or urethral opening prior to urine collection. By not mandating this step we obtained a more accurate representation of the bacterial community associated with all aspects of the lower urinary tract. Bacteria in this environment were considered sources of UTI, given that UTIs are initiated by fecal ascension up the urethra<sup>16</sup>. Further, transient exposure of *E. coli* intracellular bacterial communities (IBCs) to vaginal *Gardnerella vaginalis* induces *E. coli* egress and subsequent UTI<sup>17</sup>, which highlights the importance of profiling the urethra and labial surfaces as UTI reservoirs.

With regards to antibiotic efficacy, prophylaxis appeared to be associated with a transient decrease in bacterial load that returned to pre-prophylaxis levels following cessation of therapy. Other studies suggest 50% of women will develop symptomatic UTI within 3 months of discontinuing prophylaxis<sup>18</sup>, indicating the long-term effects of prophylaxis are ineffective for these patients.

Surprisingly, we noted a significant decrease in the levels of urinary interleukin-6 (IL-6) in RUTI patients relative to healthy controls. The suggestion that IL-6 is depressed in patients raises two possibilities, the first being a genetic basis for the lack of response, and the second that bacteria are producing molecules that inhibit the immune response. A recent study supports this, whereby *E. faecalis* suppressed NF- $\kappa$ B-driven responses in macrophages and promoted polymicrobial catheter-associated UTI<sup>9</sup>. Of note, the secreted molecule(s) responsible for this effect has yet to be identified.

Moving forward, we explored the potential for *E. faecalis* to mask the host's perception of UTI in **Chapter 4**, which may contribute to its ability to influence the development of polymicrobial UTI, through the use of mass spectrometry. Our target list included a selection of classic neurotransmitters, biogenic amines, amino acids, and a variety of neuromodulators. In doing so, we identified the production of tyramine by *E. faecalis* under conditions simulating human urine. Given high levels of trace amine-associated receptor 1 (TAAR1) within the urothelium<sup>19,20</sup>, we theorise that this activity is biologically relevant, and that the bladder is able to respond to this bacterial production of tyramine.

Throughout this thesis we have referenced human studies, where possible; however, when human studies were not available, or were in contrast with animal studies, we have attempted to highlight that information. This is because of the extent of species variation within the systems activated by neuroactive substances. For example, the  $\beta$ -adrenoceptor ( $\beta$ -AR) subtype involved in detrusor relaxation varies between species; humans and pigs rely on  $\beta_3$ -AR for mediation of detrusor relaxation, rabbits on  $\beta_2$ -AR, and rats on all three subtypes<sup>21</sup>. Similarly, in a rat model of bladder obstruction, expression of  $\alpha_1$ -AR subtypes differed between control and obstructed animals;  $\alpha_{1A}$ -AR was the predominant subtype in healthy rats and  $\alpha_{1D}$ -AR predominant in obstructed rats<sup>22</sup>. Yet in humans with bladder obstruction there is no change in  $\alpha_1$ -AR subtype expression relative to healthy controls<sup>23</sup>. Given that  $\alpha_1$ -AR subtypes are characterised by agonist affinity<sup>24</sup>, such changes have the ability to significantly alter the host response. Species variation is also noted for trace amine-associated receptors (TAARs); human TAAR1 is responsive to tyramine,  $\beta$ -PEA, and DA, yet unresponsive to NE, EPI, or 5-HT<sup>25-27</sup>. In contrast, TAAR1 from the rhesus



monkey responds to tyramine,  $\beta$ -PEA, and DA in a similar manner to the human TAAR1, yet also responds to NE and 5-HT<sup>28</sup>. Anatomically, muscularis mucosae is present in the human and guinea pig bladder, yet is not morphologically evident in the rat, rabbit, cat, or dog<sup>29-31</sup>. Although, these differences may seem small, their impact is potentially huge when considering that urine contains over 3000 metabolites<sup>32</sup>, many of which modulate the expression and effects of others.

In principle, the ideal animal model for bacteria-bladder interactions is a “unicorn”; the immense complexity associated with urinary bladder signaling, suggests this is unlikely to exist. Animal models are useful, and indeed have been referenced throughout this thesis; yet the application of these models to the study of microbiota and/or bacterial-host interactions within the urinary tract should be considered carefully.

## 5.1 Future directions

Alternatively, we suggest the use of human bladder tissue in Ussing chamber systems. In brief, Ussing systems consist of a chamber and perfusion system, the chamber is filled with a physiologically relevant solution and separated by an epithelial or tissue layer. Each hemichamber is distinct and corresponds to either the luminal or serosal surface of the epithelium or tissue layer. The epithelial response to electrical, chemical or mechanical stimuli can then be measured as diffusion between the chambers, basolateral or apical release of chemicals, and alterations in electrophysiology, including transmembrane voltage, transepithelial electrical resistance (TER), and short circuit current. Ussing chambers have been effectively applied to study of the urothelium, providing measurements of ATP release, membrane capacitance, and alterations in barrier function induced by multiple stimuli, such as distension and osmotic stress<sup>33-36</sup>. Ussing systems have also been used to study bacterial attachment to the gut mucosa following addition of chemicals to the luminal or contraluminal hemichambers.

Notably, addition of tyramine to the luminal surface, at concentrations ranging from 10 to 1000  $\mu$ M, dose-dependently increased *E. coli* attachment to the mucosa by 4- to 16-fold, an effect that was mediated through its sympathomimetic properties<sup>37-39</sup>. Further, this effect is not species specific; the same has been demonstrated for *Salmonella enterica*

serovar cholerasuis using *ex vivo* Peyer's patches isolated from the porcine jejunum<sup>40</sup>. Interestingly, our targeted metabolomics data (**Chapter 4**) indicates enterococci produce similar concentrations of tyramine in artificial urine, which raises questions about whether this relationship exists in the bladder. This mechanism may explain the positive correlation between urinary enterococcal load and non-*E. coli* uropathogens noted in **Chapter 3**. Thus, similar experiments should be completed using human bladder tissue and these should include pre-treatment of the luminal aspect with enterococci followed by the addition of other uropathogens. Indeed, the mechanisms and pathogenesis of polymicrobial UTI are poorly understood; study of the role enterococci play in this condition would fill this void.

Acquisition of human bladder tissue is understandably difficult; however, these experiments do not require large tissue segments, and could be sourced through urologists completing various surgeries or organ donation upon death. The disclaimer being that surgical intervention is not required in those with healthy urinary tracts, thus results would need to take into consideration the reason for surgical intervention. For instance, expression of cadherin-catenin complexes is highly variable in urothelial cell lines isolated from urinary cancers. Given that these complexes are involved in adherens junction (AJ) formation<sup>41</sup>, their dysregulation may be a sign of bladder mucosal remodeling. As discussed in the introduction, bladder mucosa remodelling occurs in response to chronic UTI, and is characterised by loss of terminal UC differentiation and impaired barrier function<sup>42-46</sup>. Clearly, responses elicited from such tissues may not correspond with the response of a healthy bladder.

Within **Chapter 2**, we explored the antimicrobial properties of ibuprofen (IBU) and one of its major metabolites, carboxyibuprofen (CIBU); however, we were unable to link IBU with direct antimicrobial properties *in vitro*. Neither IBU nor CIBU altered growth of *E. coli* or *E. faecalis* at biologically relevant concentrations, nor did these molecules effect bacterial attachment to urothelial cells. Therefore, we support the conclusions by other groups that IBU's *in vivo* anti-UTI effects are likely mediated through its anti-inflammatory properties<sup>42,47-49</sup>. The contribution of prostaglandins (PGs) to UTI is poorly understood, yet the increase in clinical cure rate associated with inhibition of

cyclooxygenases (COX)<sup>47,49-51</sup>, and decrease in intracellular colonisation following COX-2 inhibition<sup>42</sup>, indicates PGs play a key role in UTI pathogenesis. Indeed, PGE<sub>2</sub> release is increased following urothelial exposure to pyocyanin, lipopolysaccharide (LPS), and type-1 fimbriae<sup>52,53</sup>. Furthermore, urinary concentrations are increased in patients with UTI<sup>54,55</sup>; however, PG concentrations have not been assessed in the urine of patients with RUTI between symptomatic episodes. As we have stored aliquots of urine from the clinical study described in **Chapter 3**, future studies have the capability to measure urinary PG, and can correlate the bacteriological data with urinary PG concentrations. Identification of increased urinary PGs in paediatric patients with RUTI relative to healthy controls may provide the impetus for clinical study of IBU for prevention of RUTI, rather than just symptomatic therapy.

The assessment of urinary tyramine in the samples from **Chapter 3** is also of interest, particularly in samples *E. faecalis* was isolated from. Such a correlation, in combination with our *in vitro* metabolomics data from **Chapter 4**, would support our proposed theory of tyramine-mediated enterococcal contribution to polymicrobial UTI. Assessment of the host response to tyramine is also of interest, and members of the Burton lab are already looking into the expression of monoamine oxidases in response to bacterial exposure. Together with experiments using the Ussing system, future studies may be able to elucidate mechanisms of enterococcal-influence in polymicrobial infections in UTI. This understanding will provide groundwork to expand to other sites of common enterococcal infection, and will expand the understanding of enterococcal-host interactions as a whole.

## 5.2 Concluding remarks

Although the focus of this thesis was not polymicrobial UTI, our results suggest *E. faecalis* may contribute more to this state than previously thought. We identified enterococci in a large proportion of our clinical samples and further a positive correlation between *E. faecalis* and non-*E. coli* uropathogens, which our *in vitro* data suggests may be mediated through enterococcal production of tyramine. We also identified nitrofurantoin-induced increases in enterococcal attachment to the urothelium. Taken together, these data raise interesting questions about the application of nitrofurantoin treatment of enterococcal UTI and the efficacy of antibiotic prophylaxis for RUTI.

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**Title:** The microbiome of the urinary tract[mdash]a role beyond infection

**Author:** Samantha A. Whiteside, Hassan Razvi, Sumit Dave, Gregor Reid, Jeremy P. Burton

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## Appendix B: Ethical approval for clinical study



**Western  
Research**

Research Ethics

**Western University Health Science Research Ethics Board  
HSREB Amendment Approval Notice**

**Principal Investigator:** Dr. Sumit Dave

**Department & Institution:** [REDACTED]

**HSREB File Number:** 102817

**Study Title:** Recurrent urinary tract infections in children: Bacterial identification, antibiotic susceptibility profiling and cytokine levels associated with antibiotic prophylaxis

**Sponsor:**

**HSREB Amendment Approval Date:** June 23, 2014

**HSREB Expiry Date:** June 30, 2015

**Documents Approved and/or Received for Information:**

Document Name	Comments	Version Date
Revised Western University Protocol	Western protocol revised clean	
Assent	Assent revised clean	
Letter of Information & Consent	LOI Revised clean	

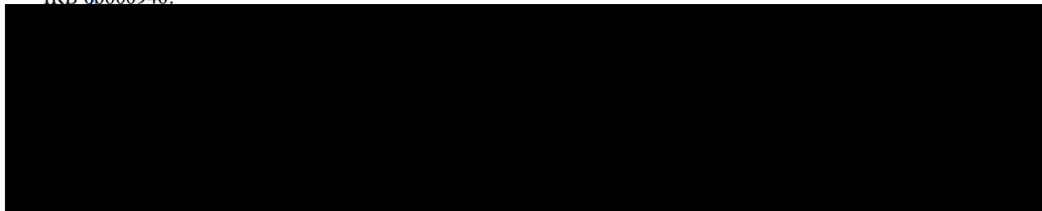
The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the amendment to the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review. If an Updated Approval Notice is required prior to the HSREB Expiry Date, the Principal Investigator is responsible for completing and submitting an HSREB Updated Approval Form in a timely fashion.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.



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**LAWSON HEALTH RESEARCH INSTITUTE****FINAL APPROVAL NOTICE**

RESEARCH OFFICE REVIEW NO.: R-12-387

PROJECT TITLE: Recurrent urinary tract infections in children: Bacterial identification, antibiotic susceptibility profiling and cytokine levels associated with antibiotic prophylaxis

PRINCIPAL INVESTIGATOR: Dr. Sumit Dave

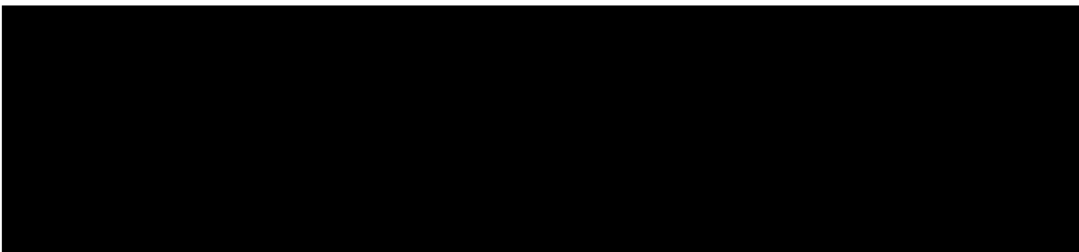
DATE OF REVIEW BY CRIC: September 18, 2012

Health Sciences REB#: 102817

Please be advised that the above project was reviewed by the Clinical Research Impact Committee and the project:

**Was Approved**




**PLEASE INFORM THE APPROPRIATE NURSING UNITS, LABORATORIES, ETC. BEFORE STARTING THIS PROTOCOL. THE RESEARCH OFFICE NUMBER MUST BE USED WHEN COMMUNICATING WITH THESE AREAS.**



cc: Administration

## Appendix C: Clinical study documentation

### Letter of Intent

		
<p><b><u>Recurrent urinary tract infections in children: Bacterial identification, antibiotic susceptibility profiling and cytokine levels associated with antibiotic prophylaxis</u></b></p>		
<p><b>Investigator:</b>     <b>Dr. Sumit Dave</b></p>		
<p>The pronouns 'you' and 'your' should be read as referring to the participant rather than the parent/guardian/next of kin who is signing the consent form for the participant.</p>		
<p><b>Purpose of Study</b></p> <p>The purpose of the study is to identify the changes in bacterial profiles and antibiotic susceptibilities and disease severity in children receiving antibiotic prophylaxis as a treatment for recurrent urinary tract infection. Drs. Sumit Dave and Gregor Reid will administer the study at the Children's Hospital, University of Western Ontario and Lawson Health Research Institute.</p>		
<p><b>Procedures</b></p> <p>Pediatric patients between the ages of 3 and 15 years are invited to participate in this study if they have been diagnosed with a recurrent urinary tract infection and prescribed antibiotics. Participants will have either received antibiotics before, or be receiving antibiotics for the first time. The study is also going to include approximately 20-30 participants who do not experience urinary tract infections. This research study will only involve those who choose to take part. This letter of information and consent form describe the study so you can make an informed decision on participating. Please take time to make a decision and if necessary, discuss this proposal with your doctor, family members and friends. Please feel free to ask questions if anything is unclear or there are words or phrases you do not understand. You have been asked to participate because you have been diagnosed with recurrent urinary tract infection and prescribed antibiotics.</p>		
<p>If you agree to participate, you will be asked to come for a visit. On the day of the first visit (baseline appointment) you will be asked for a urine sample, undergo uroflowmetry and to fill in the dysfunctional voiding scoring system (DVSS) questionnaire, possibly with the assistance of a parent or guardian.</p>		
Version Date: June 6, 2014	Page 1 of 5	Initials: _____

You will be provided with a prescription for antibiotics and instructions on taking them. If you are in the group of the study that does not experience urinary tract infections you won't have to take antibiotics, but you will be asked to participate in other study-related procedures (such as giving a urine sample and filling out a questionnaire). There will be follow up visits at months 3, 6, 9, and 12; as well as any additional times that you experience a urinary tract infection. Each visit will involve similar examinations as the baseline appointment.

### **Number of Participants**

This study hopes to enroll 75 participants; 25 patients new to antibiotics, 25 patients receiving antibiotics for a second time, and 25 participants who do not experience urinary tract infections.

### **Participant Inclusion and Exclusion Criteria**

Participants will be included if they have been diagnosed with recurrent urinary tract infection and require antibiotics.

Participants will be excluded if they are unable to make an informed decision, are severely immune compromised, have an abnormal urinary tract or any other condition that may influence an outcome.

### **Description of Research**

As a participant in this study you will be asked to provide the following;

- 1) Urine sample
- 2) Fill in the DVSS questionnaire

### **Time Requirements**

The collection of urine samples takes 5-10 minutes and will be completed during a regularly scheduled appointment with Dr. Dave.

### **Risks**

There are no known risks associated with participating in this study.

### **Benefits**

There are no known direct benefits for participating in this study, however it is hoped that the information gathered will benefit future patients who also suffer from recurrent urinary tract infections.

### **Voluntary Participation**

Your participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or you may withdraw from the study at any time with no effect on your future healthcare. Samples and information collected before the date of withdrawal will not be excluded from the study.

### **Participation in concurrent or future studies**

While the likelihood of this study interfering with other studies is minimal, please inform Dr. Dave immediately if you are participating in or plan to be involved in another study. He will then determine if it is appropriate for you to continue participation in this study.

### **Use of Data**

The urine samples will become the property of the researchers and once you have provided them you will not have further access to them. They will be used and retained by Dr. Reid for research purposes; however, specimens retained by Dr. Reid will not be linked to personal information. Data will be kept for 7 years and will then be destroyed.

### **New Findings**

If, during the course of this study, new information becomes available that may relate to your willingness to continue to participate, this information will be provided to you by the investigator.

### **Confidentiality**

Your privacy will be respected. All medical records and research materials in which you are identified will be kept confidential and will not be made publicly available, unless required by applicable laws or regulations. No personal identifiers will be sent off site. If the results of this study are published, your name will not be used and no information that discloses your identity will be released.

Representatives of The University of Western Ontario Health Sciences Research Ethics Board may contact you or require access to your study-related records to monitor the conduct of the research. All electronic data collected for this study will be stored on the hospital drive which is a secure network and access will only be granted to authorized personnel. All hard copies will be stored in a locked office.

All participants will be given a study number. Only that number and your initials will be used on any study related documents. The master list will be held separate from the data in a secure research office at Children's Hospital on a confidential computer server. Your family doctor will be notified of your participation in this research project unless you do not give permission.

We cannot guarantee that the results of this study will be made accessible to you, but if you would like to be informed of the outcome of the study you are asked to provide current contact information.

### **Contacts**

If you have any questions about your rights as a research participant or the conduct of the study you may contact Dr. David Hill, Scientific Director, Lawson Health Research Institute at

If you have any questions during the study, experience a side effect from sample collection or wish to withdraw from the study at any time, you may contact Dr. Sumit Dave, Principal Investigator at



# Western



**Recurrent urinary tract infections in children: Bacterial identification, antibiotic susceptibility profiling and cytokine levels associated with antibiotic prophylaxis**

**Consent Form**

I have read the Letter of Information, have had the nature of the study explained to me and I agree/I agree for my child to participate. All questions have been answered to my satisfaction and I understand that once this form is signed I will receive a photocopy to keep.

\_\_\_\_\_  
Printed Name of Participant

\_\_\_\_\_  
Signature of Participant

\_\_\_\_\_  
Date (DD-MON-YYYY )

\_\_\_\_\_  
Printed Name of Legally  
Authorized Representative

\_\_\_\_\_  
Signature of Legally  
Authorized Representative

\_\_\_\_\_  
Date (DD-MON-YYYY )

\_\_\_\_\_  
Printed Name of Person  
Obtaining Consent

\_\_\_\_\_  
Signature of Person  
Obtaining Consent

\_\_\_\_\_  
Date (DD-MON-YYYY )



# Western



**Recurrent urinary tract infections in children: Bacterial identification, antibiotic susceptibility profiling and cytokine levels associated with antibiotic prophylaxis**

**Interest in Study Outcome**

Please indicate whether you would like to be informed of the outcomes of this study:

☐ Yes (provide contact information below) ☐ No

Address: \_\_\_\_\_  
 \_\_\_\_\_  
 \_\_\_\_\_  
 \_\_\_\_\_

Please indicate whether your family doctor may be notified of your participation in this research

☐ Yes ☐ No



## Letter of Assent



### **Recurrent urinary tract infections in toilet trained children: Alterations to bacterial profile and antibiotic susceptibilities associated with the use of prophylactic antibiotics**

**Investigator: Dr. Sumit Dave**

#### **Why are you here?**

Dr. Sumit Dave wants to discuss a study with you about children with recurrent urinary tract infections. Dr. Dave is wondering if you would like to participate in this study because you have a recurrent urinary tract infection and are going to be taking antibiotics to treat your infection.

#### **Why are we doing this study?**

Dr. Dave, along with researchers at the Lawson Health Research Institute, want to know if your antibiotic treatment is changing the bacteria that are in your urine. They also hope to determine if your treatment is working.

#### **What will you have to do?**

If you agree to participate in the study, this is what will happen:

1. You and your parent/guardian will answer some questions.
2. You will start taking antibiotics according to the directions Dr. Dave has given you. These are the same antibiotics you will be getting even if you decide not to be involved in the study. If you are going to be in the study group that does not have recurrent urinary tract infections you will not be taking antibiotics but you will be asked to participate in other parts of the study, like giving a urine sample and filling out a questionnaire.
3. Every 3 months you will give a urine sample, the doctors will then study your urine. The urine sample will be collected during an appointment with Dr. Dave and will involve you peeing in a cup.
4. If you and your parent/guardian are willing, you will also give a urine sample when you are experiencing urinary tract infection symptoms during the study.

No part of this study should cause you pain or make you physically uncomfortable.

#### **How does this study benefit you?**

The study may not directly benefit you, however, the results of this study may help other children like you with recurrent urinary tract infections in the future.

#### **What about questions?**

You can ask questions anytime, now or later. You can talk to your doctors, family, or friends, it is up to you. Feel free to ask questions if anything is unclear or there are words or phrases you do not understand.

#### **Do you have to participate in the study?**

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Initials: \_\_\_\_\_

Your participation in this study is voluntary. You may choose not to participate or refuse to answer any questions; there will be no negative consequences. If you change your mind and decide you no longer want to participate, you may withdraw from the study at any time.



### Assent

**I have read the assent form, have had the study explained to me and agree to participate. All my questions have been answered and I understand that once this form is signed I will receive a copy to keep.**

\_\_\_\_\_  
Print Name of Child

\_\_\_\_\_  
Signature of Child

\_\_\_\_\_  
Age

\_\_\_\_\_  
Date

\_\_\_\_\_  
Signature of Person Obtaining Assent

\_\_\_\_\_  
Date

## Clinic Intake form



***PLEASE NOTE: IF THESE FORMS ARE NOT FILLED  
OUT IN FULL AND BROUGHT TO YOUR CONSULTATION  
APPOINTMENT, YOUR CHILD WILL NOT BE SEEN!***

**DAYTIME WETTING**

Does your child wet his/her clothes during the day?.....Yes\_\_\_\_ No\_\_\_\_  
 Has your child **ever** been dry during the day?.....Yes\_\_\_\_ No\_\_\_\_  
 If yes, for how long \_\_\_\_\_ (weeks/months/years)  
 And at what age \_\_\_\_\_ (years)  
 On how many days a week does your child wet during the day \_\_\_\_\_ (days per week)  
 How many times a day does your child wet \_\_\_\_\_ (times per day)  
 Is the clothing usually damp? .....Yes\_\_\_\_ No\_\_\_\_  
 Is the clothing usually wet?.....Yes\_\_\_\_ No\_\_\_\_  
 Does urine dribble constantly?.....Yes\_\_\_\_ No\_\_\_\_  
 Does your child wet his/her clothes immediately after  
 going to the toilet?.....Yes\_\_\_\_ No\_\_\_\_  
 Does your child notice when he/she wets?.....Yes\_\_\_\_ No\_\_\_\_

**NIGHTTIME WETTING**

Does your child wet the bed (or diapers) during the night?.....Yes\_\_\_\_ No\_\_\_\_  
 Has your child **ever** been dry at night?.....Yes\_\_\_\_ No\_\_\_\_  
 If yes, for how long \_\_\_\_\_ (weeks/months/years)  
 And at what age \_\_\_\_\_ (years)  
 On how many nights a week does your child wet? \_\_\_\_\_ (nights per week)  
 Is the bed usually damp?.....Yes\_\_\_\_ No\_\_\_\_  
 Is the bed usually wet?.....Yes\_\_\_\_ No\_\_\_\_  
 Does your child wake up to go to the toilet?.....Yes\_\_\_\_ No\_\_\_\_  
 Does your child wake up after wetting the bed?.....Yes\_\_\_\_ No\_\_\_\_  
 Is your child a deep sleeper ie. Difficult to wake up?.....Yes\_\_\_\_ No\_\_\_\_  
 Has other members of your family wetted? (Day/night).....Yes\_\_\_\_ No\_\_\_\_

**TOILET HABITS**

How many times a day does your child void (on average) \_\_\_\_\_ (times/day)  
 How long can your child manage without going to the toilet (shopping, car trips etc) \_\_\_\_\_ (hours)  
 Does your child go to the toilet by themselves when needed .....Yes\_\_\_\_ No\_\_\_\_  
 Do you have to send your child to the toilet? .....Yes\_\_\_\_ No\_\_\_\_  
 If your child wants to pee, do they have to strain at the  
 beginning or during voiding? .....Yes\_\_\_\_ No\_\_\_\_  
 When your child voids, is the stream interrupted?.....Yes\_\_\_\_ No\_\_\_\_  
 Does your child hurry and not take enough time to void?.....Yes\_\_\_\_ No\_\_\_\_

**OBSERVABLE REACTIONS**

Does your child feel a sudden urge to go to the toilet?.....Yes\_\_\_\_ No\_\_\_\_  
 When your child has to void, do they have to rush to  
 the toilet immediately?.....Yes\_\_\_\_ No\_\_\_\_  
 Does your child cross their legs, squat, sit on their  
 heels to prevent wetting?.....Yes\_\_\_\_ No\_\_\_\_  
 Does your child postpone going to the toilet for as long as possible.....Yes\_\_\_\_ No\_\_\_\_  
 If yes, in which situations? (school/play/TV etc)

Please specify: \_\_\_\_\_

**URINARY TRACT INFECTIONS**

Has your child **ever** had a urinary tract infection?.....Yes \_\_\_\_ No \_\_\_\_

If yes, how many times? \_\_\_\_\_

Has your child had urinary tract infections with fever?.....Yes \_\_\_\_ No \_\_\_\_

Has your child been treated for any illness of the urinary tract?.....Yes \_\_\_\_ No \_\_\_\_

If yes, please specify: \_\_\_\_\_

**STOOL HABITS**

Does your child have regular bowel movements?.....Yes \_\_\_\_ No \_\_\_\_

If not, how many times/week: \_\_\_\_\_

Is your child regularly constipated?.....Yes \_\_\_\_ No \_\_\_\_

Does your child soil their underwear (during the day)?.....Yes \_\_\_\_ No \_\_\_\_

Does your child soil during sleep?.....Yes \_\_\_\_ No \_\_\_\_

If yes, small amounts? (smear).....Yes \_\_\_\_ No \_\_\_\_

Or: large amounts? (stool).....Yes \_\_\_\_ No \_\_\_\_

How often does your child soil? \_\_\_\_\_ x/week \_\_\_\_\_ x/month

Has your child previously had a complete bowel control?.....Yes \_\_\_\_ No \_\_\_\_

If yes, for how long \_\_\_\_\_ (weeks/months/years)

And at what age \_\_\_\_\_ (years)

Does the soiling occur in special situations?.....Yes \_\_\_\_ No \_\_\_\_

If yes, please specify: \_\_\_\_\_

**BEHAVIOR: WETTING**

Is your child distressed by their wetting?.....Yes \_\_\_\_ No \_\_\_\_

Are you distressed because of your child's wetting?.....Yes \_\_\_\_ No \_\_\_\_

Has your child been teased because of the wetting?.....Yes \_\_\_\_ No \_\_\_\_

Are there any things your child did not do (school outings/sleepovers) because of the wetting?.....Yes \_\_\_\_ No \_\_\_\_

Does your child wet more often in stressful times?.....Yes \_\_\_\_ No \_\_\_\_

Is your child cooperative and motivated for treatment?.....Yes \_\_\_\_ No \_\_\_\_

If your child was previously dry, do you see any event that might be associated with the relapse?.....Yes \_\_\_\_ No \_\_\_\_

If yes, please specify: \_\_\_\_\_

What, in your opinion is the reason for wetting? Please specify: \_\_\_\_\_

**BEHAVIOR: GENERAL**

Does your child have difficulties in accepting rules?.....Yes \_\_\_\_ No \_\_\_\_

Is your child restless, on the go, easily distracted?.....Yes \_\_\_\_ No \_\_\_\_

Does your child have difficulty concentrating?.....Yes \_\_\_\_ No \_\_\_\_

Is your child sometimes anxious?.....Yes \_\_\_\_ No \_\_\_\_

Is your child sometimes sad, unhappy, withdrawn?.....Yes \_\_\_\_ No \_\_\_\_

Does your child have problems at school?.....Yes \_\_\_\_ No \_\_\_\_

If yes, please specify: \_\_\_\_\_

Does your child have problems in other areas?.....Yes \_\_\_\_ No \_\_\_\_

If yes, please specify: \_\_\_\_\_

## 48-hour bowel bladder diary

48 HOUR FREQUENCY/VOLUME CHART \*PATIENT NAME:\_\_\_\_\_

*THIS MUST BE FILLED OUT IN FULL OR YOU WILL NOT BE SEEN.*

FILL OUT AND BRING TO YOUR CONSULTATION APPOINTMENT

**Instructions:** Please fill out this chart on a day without school, or on a weekend/holiday, every time your child goes to the toilet, or wets. This should start one morning and continue for 48 hours. Please discuss with your child prior. You should not "send" your child to the toilet, instead they should tell you when they need to go. Have them empty into a measuring cup. Please measure the amount of urine, record it with the time of day. You do not need to "save" the urine, it may be flushed once recorded. Please note the following on the chart: Does your child strain getting started or if the stream is interrupted, if your child wets their clothes, please note the time and if they were damp or wet. If your child feels a sudden urge to go to the toilet, not this in the next column. If your child crosses their legs, squats or tries to hold back the urine, please note this with the time in the next column. Finally: please record the amount of fluid your child drinks during the day, along with the time. Thank you for your assistance!

[illegible]

## Dysfunction voiding scoring system questionnaire

This Section For Office Use Only:

Patient Identifier:

Date:

Appointment:



### Voiding Habits Questionnaire

Over the last month	Almost Never	Less than half the time	About half the time	Almost every time	Not available
1 – I have had wet clothes or wet underwear during the day	0	1	2	3	NA
2 – When I wet myself, my underwear is soaked.	0	1	2	3	NA
3 – I miss having a bowel movement everyday.	0	1	2	3	NA
4 – I have to push for my bowel movements to come out.	0	1	2	3	NA
5 – I have to go to the bathroom one or two times a day.	0	1	2	3	NA
6 – I can hold onto my pee by crossing my legs, squatting or doing the “pee dance.”	0	1	2	3	NA
7 – When I have to pee, I cannot wait.	0	1	2	3	NA
8 – I have to push to pee.	0	1	2	3	NA
9 – When I pee it hurts.	0	1	2	3	NA
10 – Parents to answer: Has your child ever experienced something stressful like the examples below?	NO (0)		YES (3)		
TOTAL					

- New baby.
- New home.
- New school.
- School problems.
- Abuse (sexual/physical).
- Home problems (divorce/death).
- Special events (birthday).
- Accident/injury.
- Others

Adapted from Farhat et al., 2000, I Urol.

## Appointment schedule letter example

<p>Dr. Sumit Dave, Paediatric Urology</p> 	 <p><b>Children's Hospital</b> London Health Sciences Centre</p>
<p>December 20<sup>th</sup>, 2013</p>	
<p><b><u>RE: UTI STUDY APPOINTMENTS</u></b></p>	
<p>Dear Parents:</p>	
<p>Please find enclosed your child's follow up appointments. As per the study they have enrolled in, Dr. Dave needs to see them every 3 months. When you come to the appointments, please have your child come with a full bladder, and prepared to give us a urine sample.</p>	
<p>If you have any questions, please call the office</p>	
<p>Yours truly,</p>	
<p><i>Sandra</i> Admin. Assistant Dr. Sumit Dave Paediatric Urology</p>	
<p><b><u>APPOINTMENTS</u></b></p> <ul style="list-style-type: none"> <li>○ March 20<sup>th</sup> @ 1:45</li> <li>○ June 19<sup>th</sup> @ 2:40</li> <li>○ September 18<sup>th</sup> @ 1:25</li> <li>○ December 11<sup>th</sup> @ 1:40</li> </ul>	



## Curriculum Vitae

**Name:** Samantha Ann Whiteside

**Post-secondary education and degrees:** University of Guelph  
Guelph, Ontario, Canada  
2007-2011 HBSc

The University of Western Ontario  
London, Ontario, Canada  
2011-2018 PhD

**Honours and awards:** Graduate Student Teaching Award  
2016

Leadership Award - Lawson Impact Awards  
2016

Province of Ontario Graduate Scholarship  
2014-2015

Earl Russell Trainee Grant in Pain Research  
2013-2014

**Related work experience:** Teaching Assistant  
The University of Western Ontario  
2012-2016

Undergraduate Summer Research Assistant  
The University of Guelph  
Summer 2010 and 2011

### **Publications:**

**Whiteside, SA,** Dave, S, Wang, P, Seney, SL, Reid, G, Burton, JP. 2018. *Enterococcus faecalis* persistence in paediatric patients treated with antibiotic prophylaxis for recurrent urinary tract infections (RUTI). Future Microbiol. {IN REVIEW}.

**Whiteside, SA,** Dave, S, Reid, G, Burton, JP. 2018 Lack of evidence that ibuprofen has antimicrobial properties to treat urinary tract infection. {IN SUBMISSION}

Bao Y, Al KF, Chanyi RM, **Whiteside SA,** Dewar M, Razvi H, Reid G, Burton, JP. 2016. Questions and challenges associated with studying the microbiome of the urinary tract. Ann Transl Med. 5(2):e1-8.

Yang L, **Whiteside SA**, Cadieux PA, Denstedt JD. 2015. Ureteral stent technology: Drug-eluting stents and stent coatings. *Asian J Urol.* 2(4):194-201.

**Whiteside SA**, Razvi H, Dave S, Reid G, Burton JP. 2015. The microbiome of the urinary tract – a role beyond infection. *Nat Rev Urol.* 12:81-90. (Cover)

Goneau LW, MacDonald KW, Huynh MJ, **Whiteside SA**, Yang L, Cadieux PA. 2012. Effects of subinhibitory concentrations of ciprofloxacin on *Staphylococcus saprophyticus* adherence and virulence in urinary tract infections. *Urotoday.com* (commentary), March 2012 (ISSN 1939-4810 – <http://www.urotoday.com/infections-1165>).

Kircanski J, Parreira VR, **Whiteside S**, Pei Y, Prescott JF. 2012. The majority of atypical *cpb2* genes in *Clostridium perfringens* isolates of different domestic animal origin are expressed. *Vet Micro.* 159:371-374.